1944

The mechanism of formation of acetylmethylcarbinol by active enzyme preparations

Noel Harden Gross
Iowa State College

NOTE TO USERS

THE MECHANISM OF FORMATION OF ACETYLMETHYLCARBINOL BY ACTIVE ENZYME PREPARATIONS

by

Noel Harden Gross

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Physiological Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

1944

TABLE OF CONTENTS

	Page
INTRODUCTION	1
HISTORICAL	2
METHODS	13
Preparation of the Enzyme Systems	13
Bacterial enzyme preparation	13
Yeast enzyme preparation	15
Animal enzyme preparation	15
Qualitative Procedures	16
Acetylmethylcarbinol	16
Quantitative Procedures	16
Acetylmethylcarbinol	16
Pyruvic acid	17
Carbon dioxide	17
Acetaldehyde	19
Lactic acid	19
Heavy carbon	20
Manometric Methods	21
Preparation of Substrates	21
Heavy carbon aldehyde	21
Pyruvic acid	23
Nitrogen	23

	Page
EXPERIMENTAL	24
Acetylmethylcarbinol Formation and O_2-uptake by Cell Suspensions of *Aerobacter*	24
Fixation of Heavy Carbon Acetaldehyde in Acetylmethylcarbinol by *Aerobacter aerogenes* Juice	28
Preparation of the juice	28
Activity of the preparations	29
Fermentation apparatus	33
Procedure	35
Summary and conclusions	38
Fixation of Heavy Carbon Acetaldehyde in Acetylmethylcarbinol by Yeast Juice	38
Preparation of the juice	38
Activity of the preparations	39
Effect of dilution on the enzyme preparation	41
Effect of freezing on activity of juice	42
Effect of concentration of substrate on the activity of the juice	46
Fermentation procedures	52
Summary and conclusions	56
Formation of Acetylmethylcarbinol by Pig Heart Juice.	57
Preparation of the juice	57
Activity of enzyme preparation	58
Summary and conclusions	60
Degradation of Heavy Carbon Acetylmethylcarbinol Formed by Yeast Juice	60
Purification of heavy carbon acetylmethylcarbinol	60
Degradation procedures	61
Results of the degradation of heavy carbon acetylmethylcarbinol	65
Acetylmethylcarbinol Formation from Various Yeast Preparations	71

Page

DISCUSSION 73

SUMMARY AND CONCLUSIONS 79

ACKNOWLEDGMENT 82

LITERATURE CITED 83

LIST OF TABLES

		Page
Table 1.	O_2-Uptake and Acetylmethylcarbinol Formation by a Cell Suspension of *Aerobacter aerogenes* (#174)	26
Table 2.	CO_2-Production from Pyruvic Acid by Juices Prepared from *Aerobacter aerogenes*	30
Table 3.	Effect of Acetaldehyde on CO_2-Production from Pyruvic Acid by *Aerobacter aerogenes*	31
Table 4.	Addition of Heavy Carbon Acetaldehyde to a Pyruvic Acid Fermentation of a Juice of *Aerobacter aerogenes*	37
Table 5.	Production of CO_2 and Acetylmethylcarbinol by Preparations of Baker's and Brewer's Yeast from Pyruvic Acid and from Pyruvic Acid Plus Acetaldehyde	40
Table 6.	Effect of Enzyme Dilution on CO_2-production and Acetylmethylcarbinol-formation	43
Table 7.	Effect of Freezing Yeast Juice on CO_2-production and Acetylmethylcarbinol Formation	45
Table 8.	Effect of Freezing on CO_2-production and Acetylmethylcarbinol Formation from Pyruvate plus Acetaldehyde by Yeast Juice	47
Table 9.	Effect of Acetaldehyde on CO_2-production from Pyruvate by Frozen Yeast Juice	49
Table 10.	Effect of Concentration of Sodium Pyruvate on the CO_2-production and Acetylmethylcarbinol Formation by Frozen Yeast Juice	50
Table 11.	Effect of Concentration of Sodium Pyruvate on the CO_2-production and Acetylmethylcarbinol Formation of Frozen Yeast Juice in the Presence of Acetaldehyde	51

		Page
Table 12.	Heavy Carbon Acetaldehyde Fixation by Yeast Juices	54
Table 13.	CO_2-Production from Pyruvate, Acetaldehyde and Pyruvate plus Acetaldehyde by a Juice from Pig Heart	59
Table 14.	Degradation of Heavy Carbon Acetylmethylcarbinol Produced from Heavy Carbon Acetaldehyde by Yeast Juice Preparation	66
Table 15.	Degradation of Acetylmethylcarbinol by Periodate Oxidation	69
Table 16.	Degradation of Heavy Carbon Acetylmethylcarbinol	70

LIST OF DIAGRAMS AND GRAPHS

		Page
Diagram 1.	CO_2 Bulb	18
Graph 1.	Oxygen Uptake by a Cell Suspension of Aerobacter aerogenes (#174)	27
Graph 2.	Effect of Acetaldehyde on CO_2 Production by Aerobacter aerogenes (Juice 2a)	32
Diagram 2.	Fermentation Apparatus	34
Graph 3.	Effect of Freezing on Activity of Yeast Juice	44
Graph 4.	Effect of Freezing on CO_2-Production and A.M.C. Formation by Yeast Juice from Pyruvate plus Acetaldehyde	48

INTRODUCTION

The mechanism of the formation of acetylmethylcarbinol has presented a controversial question for some time. Neuberg (1925) suggested that one molecule of synthetic acetaldehyde condensed with one molecule of biologically formed acetaldehyde, produced by yeast preparations from glucose or from pyruvic acid, to form acetylmethylcarbinol. Dirscherl (1930) suggested the possibility of acetaldehyde coupling with pyruvic acid prior to decarboxylation to the acetylmethylcarbinol. Silverman et al. (1940), working with juices prepared from Aerobacter aerogenes, did not find any utilization of acetaldehyde, and therefore concluded that the aldehyde did not participate in the formation of acetylmethylcarbinol. Green et al. (1942) observed an increase in acetylmethylcarbinol when acetaldehyde was added to the fermentation of pyruvate and that other homologous aldehydes gave analogous ketols.

The present investigation was undertaken for the purpose of clarifying some of the existing contradictions by the use of heavy carbon (C^{13}) acetaldehyde as a tracer.

HISTORICAL

Acetylmethylcarbinol as the precursor of diacetyl is responsible, in part, for the development of desirable flavors in many foods. This is especially true of high quality dairy products, (Michaelian et al. 1933), (Hammer 1931); and bakery goods, (Mashing 1936), (Visser't Hooft, F. and de Leeuw, F.J.G. 1935). Michaelian, Farmer, and Hammer (1933) have conducted extensive investigations to show the role of acetylmethylcarbinol and diacetyl in butter aroma. It was Van Niel, Kluyver and Derx (1929) who detected acetylmethylcarbinol in cultures of Streptococcus cremoris and suggested its importance in the flavor and aroma of butter cultures.

Acetylmethylcarbinol is widely distributed in nature. Desmots (1904) found it in cultures of Bacillus mesentericus. Harden and Walpole (1906) isolated and described both acetymethylcarbinol and 2,3-butylene glycol from glucose fermentations by Aerobacter aerogenes. Organisms which produce acetylmethylcarbinol generally form 2,3-butylene glycol.

Harden and Norris (1912) found that Bacterium lactis aerogenes (Aerobacter aerogenes) and Bacterium cloacae (Aerobacter cloacae), when grown in peptone solutions containing any one of a series of sugars, produced acetylmethylcarbinol and 2,3-butylene glycol.

Lemoigne (1919, 1923) found the carbinol to be formed by species of Proteus and by Bacillus anthracis. Neuberg and Reinfurth (1923), Kluyver et al. (1925) and Elion (1926) found the carbinol in yeast cultures.

Yamada and Kurono (1927) found many bacteria and some yeasts to form both acetylmethylcarbinol and 2,3-butylene glycol. They suggested that the presence of acetylmethylcarbinol may be used to differentiate between fermented vinegars and imitations. The fermented vinegars contained acetylmethylcarbinol, whereas the imitations did not. These investigators suggested that the quality of Sake', the Japanese national alcoholic beverage, could be determined by the amount of acetylmethylcarbinol present. Putrid Sake' contains much larger quantities of the compound. Pritzker and Jungkunz (1930) found that acetylmethylcarbinol interfered with the determination of sugar in vinegar.

Lemoigne and Monguillon (1930, 1930a) found acetylmethylcarbinol during the germination of corn, wheat, barley, flax, oats, rye and in the blood of cattle, sheep, swine, and the horse. Small amounts of the carbinol were found by Schmalfuss (1930) in human urine, and later by Schmalfuss et al. (1934) in the fresh blood of the ox and the sheep.

The sanitarian has made use of acetylmethylcarbinol in the separation of the fecal and non-fecal members of the coli-aerogenes group

of bacteria (Levine, 1921). Stahly and Werkman (1942) suggested that 2,3-butylene glycol and acetylmethylcarbinol may prove to be of practical importance in the synthesis of rubber. They suggested that the future supply of rubber of the United States may be substantially met by the conversion of corn starch to 2,3-butylene glycol by microorganisms.

From the theoretical point of view the formation of acetylmethylcarbinol represents a synthesis, i.e., the formation of a four-carbon compound from either a three-carbon compound such as pyruvic acid or from a two-carbon compound such as acetaldehyde.

Harden and Norris (1912) found that glycerol, ethylene glycol and acetaldehyde gave rise to 2,3-butylene glycol but yielded no acetylmethylcarbinol. From these data the authors suggested a carbon-to-carbon-synthesis analogous to that which occurs in the butyric fermentation of glycerol and of lactic acid.

Considerable evidence has been presented to support the view that acetaldehyde is an intermediate in the formation of acetylmethylcarbinol from glucose. Neuberg and Reinfurth (1918), Neuberg, Nord and Wolff (1920), Neuberg and Hirsch (1921), Neuberg and Ohle (1922), Neuberg and May (1923), Neuberg and Reinfurth (1923), Neuberg and Rosenthal (1924), Neuberg and Simon (1925), and Neuberg and Kobel (1929) concluded that the carbinol is formed by the condensation of one molecule of added aldehyde with one molecule of biologically formed aldehyde. Neuberg

pictures the formation of the carbinol in the following manner:

$$2CH_3COCOOH \xrightarrow{\text{carboxylase}} 2CO_2 + 2CH_3CHO$$

$$2CH_3CHO \xrightarrow{\text{carboligase}} CH_3\text{-}COCHOHCH_3$$

The first reaction is catalyzed by carboxylase. The second reaction, the condensation, Neuberg attributes to the enzyme carboligase. Normal fermentations of glucose by yeast yield no acetylmethylcarbinol or 2,3-butylene glycol. Kluyver and Donker (1925) suggested that acetaldehyde is a normal intermediate but that it is removed so rapidly that no condensation occurs. When other hydrogen acceptors were added, such as oxygen, methylene blue, and sulfur, acetylmethylcarbinol and 2,3-butylene glycol were both formed.

Reynolds and Werkman (1937) observed the formation of acetylmethylcarbinol from glucose by vigorously aerated broth cultures of Escherichia coli. This finding constituted, these authors believe, proof of the intermediate formation of acetaldehyde in the fermentation of glucose. The interference of an accessory hydrogen acceptor prevented immediate reduction of the aldehyde and resulted in its accumulation and condensation.

Hirsch (1922) and Tomiyasu (1936) working with yeast, and Gorr (1932), Stepanov and Kuzin (1934) with animal preparations have

presented confirmatory evidence of the existence of carboligase. Dirscherl and Schöllig (1938) and Tanko and Munk (1939) have questioned its existence. Dirscherl and Nahm (1940) have been able to demonstrate the conversion of acetaldehyde to acetylmethylcarbinol by physical catalysts, e.g., heat and ultraviolet light. These investigators believe that pyruvate is decarboxylated enzymatically but that the condensation of the two molecules of acetaldehyde is a purely physical reaction.

There is no general agreement as to whether at least one molecule of acetaldehyde must be biologically formed to function in the condensation to acetylmethylcarbinol as suggested by Neuberg and Kobel (1925). Elion (1926) obtained a detectable quantity of acetylmethylcarbinol after three hours from a yeast suspension in three per cent acetaldehyde. Barritt (1937) inoculated Aerobacter aerogenes and Escherichia coli into peptone water containing acetaldehyde in various concentrations, and even though growth occurred no acetylmethylcarbinol was detected.

Dirscherl (1931) found, in agreement with Neuberg and Reinfurth (1923), that the addition of acetaldehyde to yeast yielded optically active acetylmethylcarbinol. When a macerated juice was used, a racemic carbinol was formed. He suggested the possibility of pyruvic acid coupling with acetaldehyde followed by decarboxylation.

Neuberg and Dirscherl are agreed that acetaldehyde is an intermediate in the formation of acetylmethylcarbinol from pyruvic acid by yeast.

Other investigators have reported the formation of acetylmethylcarbinol from pyruvic acid, Hermann and Neuschul (1932) by Acetobacter; Johnson, Peterson and Fred (1933) by Clostridium acetobutylicum; Dirscherl and Schollig (1938) by fresh yeast, dried yeast and maceration juice; Silverman and Werkman (1941) by cell-free bacterial juice; Green et al. (1942) by extracts of minced pig heart.

Silverman and Werkman (1941) reported that a cell-free enzyme preparation from Aerobacter aerogenes converts pyruvic acid into acetylmethylcarbinol and carbon dioxide without detectable intermediate formation of acetaldehyde.

Other investigators differ as to the origin of acetylmethylcarbinol. Lafon (1932) could find no pyruvic acid nor acetaldehyde in cultures of Bacillus subtilis growing in glucose and suggested the carbinol may have originated from lactic acid or from a splitting of the glucose molecule to give the carbinol directly. Horowitz-Wlassowa and Rodionowa (1933) believed that 2,3-butylene glycol originates from the direct splitting of the glucose molecule, and that acetylmethylcarbinol is a product of the biological oxidation of the 2,3-butylene glycol. Several investigators have found data to support the theory that acetylmethylcarbinol originates from 2,3-butylene glycol by biological oxidation in the absence of a fermentable carbohydrate. Walpole (1910) demonstrated the formation of acetylmethylcarbinol by growing Aerobacter

aerogenes in a medium composed of 2,3-butylene glycol, peptone and water. Lemoigne (1923) observed a decrease of 2,3-butylene glycol and an increase in acetylmethylcarbinol in old cultures of Proteus. Donker (1926) found that Aerobacillus polymyxa grown aerobically in yeast water and 2,3-butylene glycol produced acetylmethylcarbinol. This does not eliminate the possibility of the carbinol coming from some constituent in the yeast water. Verhave (1928) found that an intensive aeration of cultures of Clostridium polymyxa or Aerobacter aerogenes produced acetylmethylcarbinol instead of 2,3-butylene glycol. Werkman (1930) was able to detect acetylmethylcarbinol in cultures of Aerobacter after two days' growth in a medium consisting of 2,3-butylene glycol, ammonium sulfate and dipotassium phosphate. Barritt (1937) found that many bacteria could oxidize 2,3-butylene glycol to the carbinol. Mickelson and Werkman (1939) found that aeration under pressure increased the yield of acetylmethylcarbinol at the expense of 2,3-butylene glycol.

There is evidence, however, that in the dissimilation of glucose the carbinol is formed first and then reduced to the glycol. Neuberg and Kobel (1925) isolated 2,3-butylene glycol from a fermentation mixture of sugar and yeast to which acetylmethylcarbinol had been added. The carbinol had disappeared from the fermented mixture. Nagelschmidt (1927) found acetylmethylcarbinol as an intermediary product of the reduction of diacetyl to 2,3-butylene glycol by yeast. Visser't Hooft

and de Leeuw (1935) observed that acetylmethylcarbinol may be converted to 2,3-butylene glycol in bread. Reynolds (1936), and Reynolds and Werkman (1937) found acetylmethylcarbinol to be reduced to 2,3-butylene glycol in the presence of glucose by the colon-aerogenes bacteria. Hammer, Stahly, Werkman, and Michaelian (1935) found that under certain conditions acetylmethylcarbinol is reduced to 2,3-butylene glycol by citric acid fermenting streptococci. Stahly, Hammer, Michaelian and Werkman (1935) report that Aerobacillus polymyxa reduces acetylmethylcarbinol to 2,3-butylene glycol. Most organisms which form acetylmethylcarbinol can reduce it to 2,3-butylene glycol.

Reynolds and Werkman (1936) using a serial analysis technique obtained data which suggested that acetic acid was acting as an intermediate compound in the formation of acetylmethylcarbinol and 2,3-butylene glycol by Aerobacter indologenes. Later, Reynolds, Jacobsson and Werkman (1937) found that acetic acid was reduced when added to a glucose fermentation. Practically all of the acetic acid reduced was accounted for by a quantitative increase in 2,3-butylene glycol. Slade and Werkman (1942) using heavy carbon acetate as a tracer found that heavy carbon 2,3-butylene glycol was formed by cell suspensions of Aerobacter indologenes. The addition of acetic acid with the heavy carbon in the carboxyl group formed 2,3-butylene glycol containing heavy carbon exclusively in the hydroxyl carbon atoms. Thus a carbon-to-carbon linkage was created by the synthesis of the carbinol. They believe this indicates the participation of acetaldehyde in the con-

densation reaction. When acetic acid containing heavy carbon in both carbons was added, a 2,3-butylene glycol containing heavy carbon equally distributed between the methyl and hydroxyl carbon atoms was obtained.

Another source of acetylmethylcarbinol reported is citric acid. Hammer and coworkers (1935) showed that the addition of citric acid to milk inoculated with Streptococcus paracitrovorous resulted in increased yields of diacetyl, acetylmethylcarbinol and 2,3-butylene glycol. Hammer (1934) tried the influence of other closely related compounds on the production of acetylmethylcarbinol but found they did not increase the yield. He tried acetone, ethylacetoacetate, acetone dicarboxylate, ethyl malonate, maleic acid, malic acid, malonic acid, oxalic acid, succinic acid, and tartaric acid. Van Beynum and Pette (1939) observed that Betacoccus cremoris in the fermentation of milk produced carbon dioxide and acetic acid, whereas acidified milk (citric acid) yielded diacetyl, acetylmethylcarbinol, 2,3-butylene glycol, acetic acid and CO_2. They suggested that in an acid medium the acetaldehyde, formed from the intermediate pyruvate, participates in a cannizzaro reaction but also is dissimilated anaerobically to give acetylmethylcarbinol and aerobically to give diacetyl.

Virtanen et al. (1940) found that fermentation of glucose and of citric acid by bacteria responsible for the aroma in butter did not yield acetylmethylcarbinol, whereas fermentations containing both substrates yielded acetylmethylcarbinol. They observed that glucose plus

a suitable hydrogen acceptor (methylene blue or quinone) yielded acetylmethylcarbinol, but citric acid under similar conditions did not yield the carbinol. Slade and Werkman (1941) studied this problem with cell suspensions of Streptococcus paracitrovorous which had been grown on citrate plus lactose. They were able to study the mechanism of the fermentation of citric acid in the absence of other carbon sources. They found that citric acid was utilized but the dissimilation proceeded through oxalacetic acid and pyruvic acid to lactic, acetic, succinic, and formic acids, CO_2 and H_2.

Evidence has been submitted indicating that acetaldehyde may increase the yields of acetylmethylcarbinol or its reduction product, 2,3-butylene glycol. Harden and Norris (1912) observed the formation of 2,3-butylene glycol by Aerobacter aerogenes, from a peptone medium containing added acetaldehyde. Hammer (1936) reported the addition of acetaldehyde to fermenting milk cultures of Streptococcus liquefaciens gave increases in acetylmethylcarbinol. The addition of homologues of acetaldehyde, although increasing the carbinol yields, did not result in the formation of the homologues of acetylmethylcarbinol. Mickelson and Werkman (1938, 1939) studied the effect of adding aldehydes and fatty acids to glucose fermentations by Aerobacter on the yields of 2,3-butylene glycol. Under acid conditions there was an increase in the yield of the glycol when acetaldehyde was added.

Silverman and Werkman (1941) were unable to show an increased yield of acetylmethylcarbinol from pyruvic acid when acetaldehyde was added

to their cell-free preparation of Aerobacter. They concluded that if acetaldehyde was necessary as an intermediate their bacterial preparation did not possess the ability to activate the added aldehyde. Stahly (1936) found the addition of acetaldehyde to a glucose medium resulted in an increased yield of acetylmethylcarbinol and 2,3-butylene glycol in fermentations by Aerobacillus polymyxa conducted either aerobically or anaerobically.

Green et al. (personal communication) have shown that the addition of acetaldehyde to pyruvic acid in the presence of washed cells of Aerobacter and Escherichia gave large increases in the yield of acetylmethylcarbinol and CO_2. Green et al. (1942) prepared a yeast juice which produced an increase in the yield of acetylmethylcarbinol when acetaldehyde was added to the pyruvic acid fermentation. They report also a juice obtained from pig heart that produced a similar effect. The latter juice produced acetylmethylcarbinol from acetaldehyde alone.

METHODS

Preparation of the Enzyme Systems

Bacterial enzyme preparation

Wiggert et al. (1940) presented a method of preparing cell-free enzyme systems from bacteria by grinding a paste of bacterial cells and powdered glass. This method differs radically from the method of Booth and Green (1938) which employs the continuous passage of a heavy suspension of bacteria between a set of revolving steel rollers on a steel basin.

Aerobacter indologenes (23 B) and Aerobacter aerogenes (174) were grown in ten-liter quantities of the following medium: 1 per cent glucose, 0.3 per cent Bacto peptone, 0.3 per cent $(NH_4)_2SO_4$, 0.5 per cent K_2HPO_4, 10 per cent tap water, pH 6.8. The medium was inoculated with a young eight hour culture (250 ml.) and incubated at 30° C. for 18-24 hours; the cells were harvested by centrifuging in a Sharples continuous centrifuge. In experiments where the intact cells were used the harvested cells were washed in distilled water and recentrifuged. In experiments where the cell-free juices were employed the centrifuged cells were not washed. In the first cell-free preparations the wet cell paste was mixed with 8 parts of finely ground glass and ground by hand in a mortar with a pestle; enough phosphate buffer (pH 5.6, 1 M) was added to make the consistency of the cell-glass mixture resemble a

stiff batter. In later experiments the proportion of cells to glass was reduced to 1:2, and the grinding was done mechanically by passing the cell-glass paste between two closely-fitting glass cones. The inner cone was so constructed that it could be filled with ice and attached to a motor and rotated. The ice serves to keep the surfaces cooled. The ground cell-glass paste was extracted with cold M/2 phosphate buffer pH. 5.6 for 20 to 30 minutes at a ratio of 1.5 ml. of liquid per gram of the original bacterial paste.

The powdered glass used was prepared from clean pieces of pyrex glass ground in a ball mill containing smooth stones or steel ball bearings. The powdered glass was passed through a set of sieves allowing a maximum particle size of 0.5 mm. to pass. The average diameter of the particles was about 2 microns.

The glass and large cell particles of the above preparation were thrown down by a short centrifugation on a Swedish angle centrifuge at approximately 3500 revolutions per minute. The clear decanted liquid was centrifuged on an air-driven Beams ultracentrifuge (Beams, 1930). The cell fragments were deposited on the wall and the clear liquid was removed by means of a capillary pipette. The period of centrifugation varied from 6 to 10 minutes with the air pressure varying from 60-80 pounds. The speed of this centrifuge, with a cup approximately 3 mm. in diameter and containing about 4.5 ml. of liquid is between 75,000 to 100,000 revolutions per minute.

The bacterial extract obtained was distinctly opalescent. The activity of this juice was maintained for some time when stored in the frozen state.

Yeast enzyme preparation

The method of Green (1942) was employed in preparing the yeast enzyme. One hundred grams of air-dried baker's or brewer's yeast were rubbed up with 300 ml. of M/15 phosphate buffer, pH 7.2, and incubated for one hour at 37° C. To this mixture was added 400 ml. of distilled water. The contents were mixed well and then centrifuged in the angle centrifuge. Thirty-eight grams of ammonium sulfate were added for each 100 ml. of supernatant fluid. The precipitate was dissolved in 300 ml. of M/15 phosphate buffer, ph. 7.2 and the ammonium sulfate precipitation repeated. The precipitate was finally dissolved in 40 ml. of 0.1 M phosphate buffer, pH. 7.2. This preparation is very dark and opalescent and will maintain its activity to produce acetylmethylcarbinol for some time if stored in the frozen state.

Animal enzyme preparation

The method of Green (1942) was employed in the preparation of the active enzyme from pig heart. Fresh pig heart was minced twice in a coarse meat grinder and washed five times with 10 volumes of water. To each 80 grams of this pressed-out mince was added 40 ml. of 0.5 M - glycerophosphate buffer, pH. 6.0, and 240 grams of crushed ice. This mixture was placed in a Waring blender and homogenized for 10 minutes. The homogenized mixture was centrifuged and the decanted cloudy supernatant was mixed with 0.5 volume of crushed ice. Acetic acid (10%) was cautiously added until pH of 4.6 was reached. The precipitate

formed was rapidly centrifuged in the cold and evenly resuspended in a mixture of 10 ml. of 0.5 M phosphate buffer, pH 6.0, and 2 ml. of 0.5 M $NaHCO_3$. The final volume was about 20 ml. This cloudy preparation maintained its activity for a considerable period at 0° C.

Qualitative Procedures

Acetylmethylcarbinol

O'Meara's (1931) test using creatine and 40% KOH was employed in the detection of acetylmethylcarbinol. In some cases when the above test was negative the Barritt (1936) test was used to determine whether any acetylmethylcarbinol had been formed. One to two ml. of the liquid were employed in this test. To this was added a small amount of creatine and a volume of 40% KOH equal to the amount of liquid used. In the presence of the carbinol a pink coloration appeared in 5 to 10 minutes.

Quantitative Procedures

Acetylmethylcarbinol

A separate aliquot of the distillate was used for this determination. The method of Stahly and Werkman (1936) was employed. To a 50 or 75 ml. aliquot were added 25 ml. of 50% $FeCl_3$ and the mixture distilled. The diacetyl formed was collected in a solution containing 5 ml. Na acetate, 2 ml. $NiCl_2$, and 2 ml. NH_2OH. The precipitate was dried and weighed as the nickel glyoxime.

Pyruvic acid

Pyruvic acid was determined manometrically by ceric sulfate oxidation. At the conclusion of the experiment an aliquot of the acidified fermentation liquor was placed in the main vessel of a Warburg cup and 0.5 ml. saturated ceric sulfate solution in N H_2SO_4 was placed in the side-arm. The contents in the side-arm were dumped into the main vessel after the temperature equilibrium had been reached. The reaction was continued for two hours with constant shaking in the water bath at 30.4° C. when the CO_2 evolved was calculated. One millimole (mM) of pyruvic acid evolves 22,400 $mm.^3$ of CO_2. Lactic acid interferes in this determination, but Silverman (1941) found no lactic acid in the samples under these experimental conditions. The presence of acetylmethylcarbinol did not interfere with the determination.

Carbon dioxide

In the early experiments, the CO_2 liberated from the fermentation was collected in CO_2-free NaOH. An aliquot of this solution was treated with H_2SO_4 and the CO_2 was collected in an ascarite tube and weighed. Later the liberated CO_2 was collected in a special bulb containing about 4 ml. of CO_2-free NaOH and weighed. This bulb (Diagram 1) consists of a series of small bulbs so arranged that the gases have a maximum of surface contact with the NaOH. Each succeeding

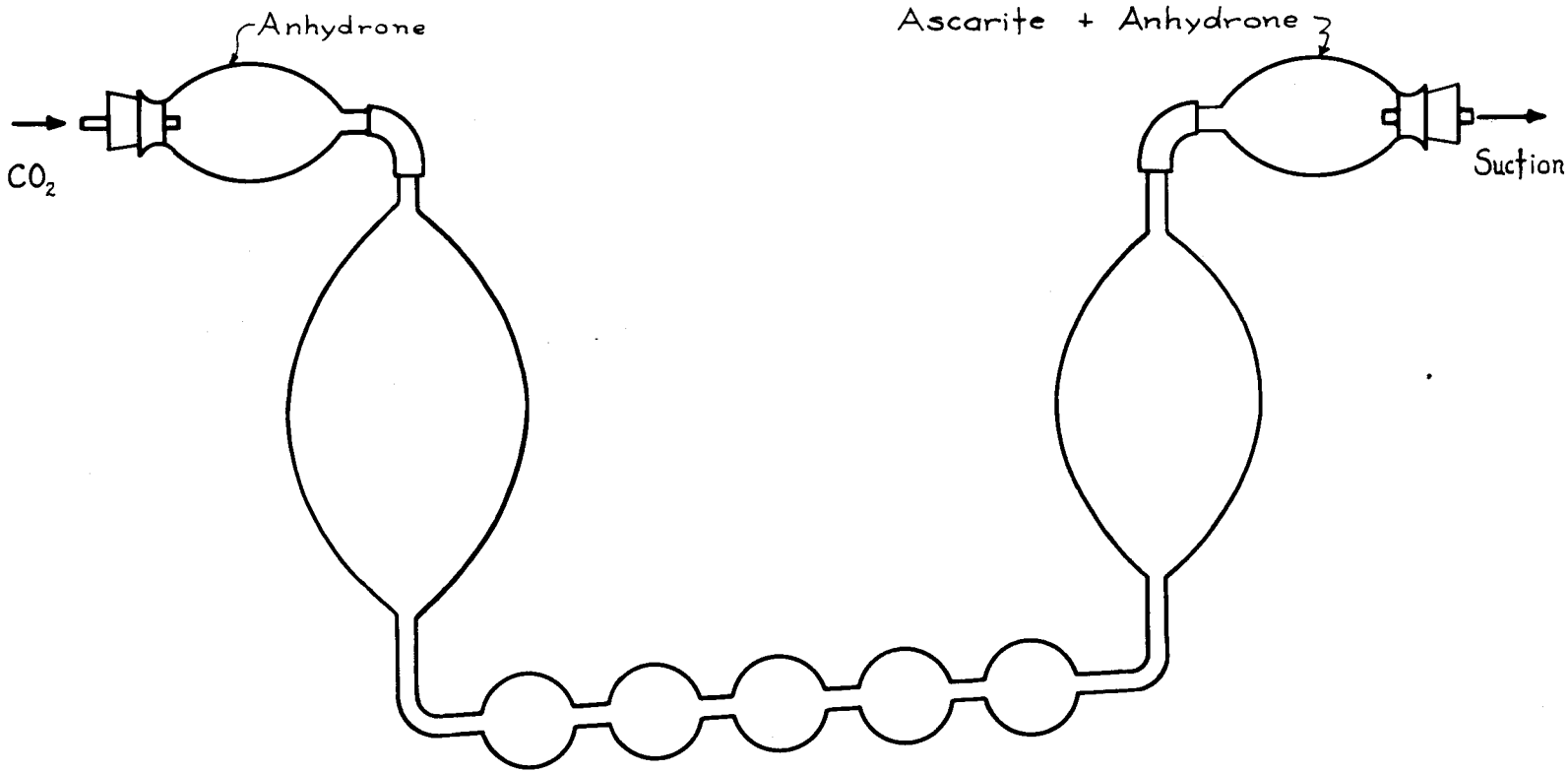


DIAGRAM 1. CO_2 BULB

bulb is slightly raised above the preceding one thus requiring the minimum of pressure to force the gases from one bulb to the other. The apparatus has an an-hydrone bulb connected at each end. The first bulb dries the incoming gases and the second retains the moisture taken up as the gases pass through the NaOH; thus the apparatus can be weighed before and after the experiment. Thus any increase of weight is due to the increase in CO_2. The total weight of the apparatus when loaded with NaOH was about 54 grams.

Acetaldehyde

The acetaldehyde was determined by the bisulfite titration of Donnally (1933). The bound bisulfite was liberated with K_2HPO_4 and the liberated bisulfite was titrated with 0.05 N iodine. K_2HPO_4 was employed because of the dilution effect of CO_2 if $NaHCO_3$ is employed when the sample is to be oxidized and tested for C^{13} content.

Lactic acid

Lactic acid was determined according to Friedemann and Graeser (1933). The lactic acid is oxidized by $KMnO_4$ to acetaldehyde and CO_2, and the aldehyde is collected in bisulfite. The CO_2 liberated is collected in the CO_2 bulb described above.

Heavy carbon

To determine the heavy carbon (C^{13}) content of the various compounds isolated from the fermentation and from the degradation reactions, each compound containing excess of the isotope was converted to CO_2. This was accomplished by the persulfate oxidation of Osburn and Werkman (1932), and the CO_2 evolved was collected in 1.5 N carbonate-free NaOH. Iodoform, which is not readily oxidized by this procedure, was oxidized by the method of Friedemann and Kendall (1929).

The CO_2 was then liberated from the alkali with 4 N lactic acid and the C^{13} content determined by the mass spectrometer as described by Nier (1940). In the first experiments the CO_2 was precipitated as the Ba salt by the addition of $BaCl_2$. The $BaCO_3$ was washed several times, centrifuged, and dried at 110° C. The carbonate was then treated with 4 N lactic acid and the liberated CO_2 was analyzed by the mass spectrometer. In later experiments the Ba salt step was omitted because of the amount of CO_2 accumulated from the air during the process of making, washing and drying the $BaCO_3$. It was found that from 4.3 to 12.5 mg. of $BaCO_3$ were formed during this process from the CO_2 from the air.

The original enriched CO_2 used in preparing the C^{13} aldehyde was obtained in this laboratory from the oxidation of methane which had been concentrated in a thermal diffusion column as described by Nier and Bardeen (1941). It was found by Nier and Gulbransen (1939) that all

naturally occurring materials contain approximately 1.09 per cent C^{13}. The per cent C^{13} is an expression of the ratio of C^{12} to C^{13} and is not an expression of the actual amount. Since the aldehyde was the only reagent containing an excess of the isotope, the presence of C^{13} in excess of 1.09 ± 0.02 per cent in any compound isolated from the experiment indicates the C^{13} has originated from the aldehyde added.

Manometric Methods

The activity of each juice was determined by the manometric technique of Barcroft and Warburg according to Dixon (1934). In general, 0.8-1.5 ml. of the juice together with the phosphate buffer at the desired pH were placed in the large vessel of the cups. The substrate was placed in the side-arm. The cups were flushed with N_2 Nitrogen and placed in the water bath. After the cups had come to temperature equilibrium, the contents of the side-arm were dumped into the main vessel. Readings were taken at various intervals of the changes of gas pressure. After two hours' or more incubation, the contents of the cups were transferred to a test tube and creatine and KOH were added to test for the presence of acetylmethylcarbinol.

Preparation of Substrates

Heavy carbon aldehyde

Acetaldehyde containing C^{13} in both carbon atoms was synthesized by a modification of the method of Cramer and Kistiakowsky (1941).

The reactions are as follows:

$$BaC^{13}O_3 + Mg \xrightarrow{700^\circ C.} BaC_2{}^{13}$$

$$BaC_2{}^{13} + H_2O \longrightarrow C_2{}^{13}H_2$$

$$C_2{}^{13}H_2 + H_2O \xrightarrow[Fe_2(SO_4)_3]{HgSO_4} C^{13}H_3.C^{13}HO$$

All materials used in the preparation of the acetylide must be perfectly dry. Fifty mM $BaCO_3$ and one mole Mg(40-80 mesh) were ground carefully in a mortar, and the mixture placed in the center of an iron pipe 1-3/8 inches in diameter. Dry hydrogen gas was passed through the tube for several minutes, and the tube was then placed in a previously heated oven at about 700° C. until it reached a red heat. The heating was continued for 5 to 10 minutes longer. Hydrogen was continuously passed through during this procedure, and the tube was then cooled in running water.

The BaC_2-Mg mixture was placed in a dry Claissen flask which had been previously swept out with nitrogen. A salt-ice bath was placed about the flask, and then 60 ml. of water were added slowly to the mixture during vigorous stirring. After the addition of the water, the mixture was warmed gently until three liters of gas were obtained. The acetylene and hydrogen gases were collected in a three-liter flask over water, and then passed through a sintered glass disk which was immersed in 100 ml. of the catalytic solution (2 gm. $HgSO_4$ plus 4.2 gm.

$Fe_2(SO_4)_3$. 9 H_2O plus 5 gm. H_2SO_4 plus water to 100 ml.) The solution was placed in a boiling water bath during the passage of the acetylene. The aldehyde was passed through a reflux condenser, placed above the catalytic solution, into 30 ml. of five per cent $NaHSO_3$. Because 10 per cent of the acetylene escapes conversion to the aldehyde, the gas from the bisulfite tower was collected and again passed through the hot catalytic solution. The yield of acetaldehyde was 58.4 per cent of theoretical. The acetaldehyde was stored as the bisulfite in the refrigerator. The acetaldehyde was liberated with K_2HPO_4 and distilled into ice cold distilled CO_2-free H_2O just before employing it in the experiments.

Pyruvic acid

Freshly vacuum distilled pyruvic acid was employed. The distillate was stored in the frozen state. In some experiments the desired amount of frozen pyruvic acid was weighed into a volumetric flask and neutralized with CO_2-free NaOH. One drop of brom-thymol blue was added. In other experiments the frozen pyruvic acid was weighed and mixed with the acetaldehyde before neutralization when smaller volumes were desired.

Nitrogen

The nitrogen used was passed over copper filings at 350° C., then through alkaline pyrogallate solution.

EXPERIMENTAL

Acetylmethylcarbinol Formation and O_2-uptake by Cell Suspensions of *Aerobacter*

The purpose of these experiments was to study the O_2-uptake and the formation of acetylmethylcarbinol by cell-suspensions of the genus *Aerobacter*.

The cells for these experiments were harvested from 18 hour broth cultures of *Aerobacter aerogenes* (#174), *Aerobacter indologenes* (#23 B), and *Aerobacter aerogenes* (#2A4) by centrifugation in a Sharples centrifuge. These cultures were obtained from the Department of Bacteriology, Iowa State College. The cells were produced in a medium consisting of: 1 per cent glucose, 0.3 per cent Bacto peptone, 0.3 per cent $(NH_4)_2SO_4$, 0.5 per cent K_2HPO_4, and 10 per cent tap water. The final pH was 6.8. The phosphate was sterilized separately and added after the medium had become cool. These cells were washed twice with distilled water and collected by centrifugation in a Swedish angle centrifuge. The cells were suspended in distilled water (40 per cent suspension) and stored in the refrigerator. Before use the cells were diluted to a 10 per cent suspension.

The experiment was performed manometrically according to the directions of Dixon (1934). Each cup contained 0.8 ml. phosphate

buffer (0.5 M pH 5.6), 0.5 ml. of the suspension of cells, 0.3 ml. 20% NaOH (in center well to absorb the CO formed), 0.2 ml. of the substrate and enough distilled water to make a total of 2.3 ml. The cell suspension was placed in the side arm and dumped into the main vessel when the temperature of the contents of the cup reached that of the water bath. Table 1 records the mm^3 of O_2 taken up during the process of the fermentation by Aerobacter aerogenes (#174). At the end of the incubation period the cup contents were dumped into test tubes and a qualitative test made for acetylmethylcarbinol.

The total O_2-uptake (Table 1) from pyruvate was 1178 mm^3 while that from pyruvate plus acetaldehyde was only 821 mm^3. This would indicate an inhibition of O_2-uptake from pyruvate in the presence of acetaldehyde. The O_2-uptake from acetaldehyde alone was 246 mm^3 which should, if there were no inhibition, be added to the 1178 mm^3 obtained from the pyruvate.

The rate of O_2-uptake from glucose (Graph 1) was much greater than that from pyruvate. The rate of O_2-uptake from pyruvate was much greater than that from pyruvate plus acetaldehyde during the first 160 minutes of the experiment but it rapidly increased during the latter part of the experiment. The O_2-uptake from acetaldehyde alone was much lower than any other substrate employed.

The relative amounts of acetylmethylcarbinol formed in the fermentation are recorded in Table 1. The cells on glucose and pyruvate yielded a considerable amount of acetylmethylcarbinol. On

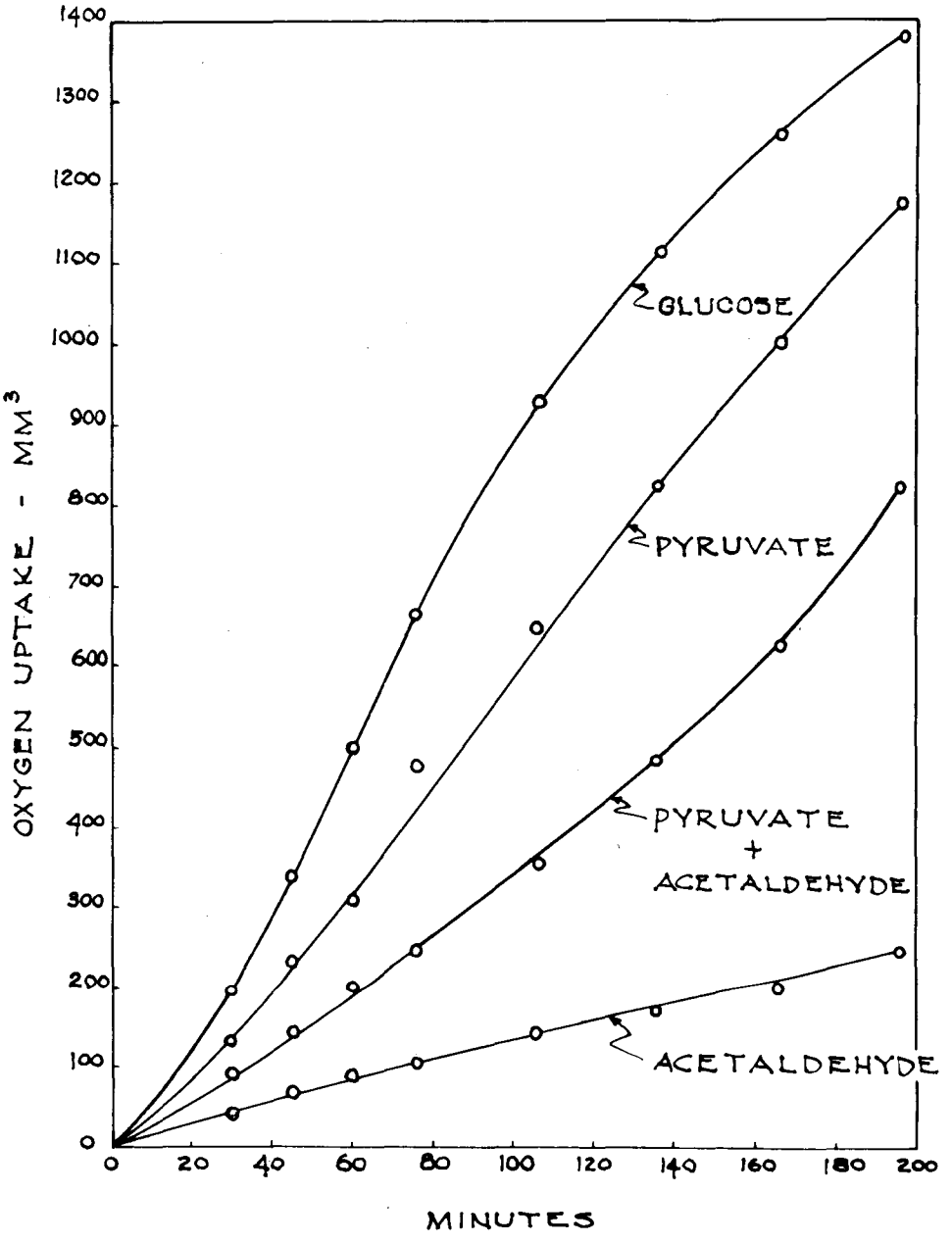
Table 1.

O_2-Uptake and Acetylmethylcarbinol Formation by a Cell Suspension of *Aerobacter aerogenes* (#174)

Time	O_2-uptake - mm^3			
	Glucose 0.01M	Pyruvate 0.01M	Acetaldehyde 0.01M	Pyruvate 0.01M Acetaldehyde 0.01M
30	199	132	47	92
45	342	232	71	149
60	498	310	92	201
75	660	476	103	245
105	926	644	142	355
135	1119	823	175	486
165	1257	997	204	627
195	1389	1178	246	821
Acetyl methyl-carbinol	+++	+++	-	+

GRAPH #1

OXYGEN UPTAKE BY A CELL SUSPENSION
OF AEROBACTER AEROGENES (# 174)



acetaldehyde as substrate, there was no carbinol formed and only a trace was evident in the fermentation containing both pyruvate and acetaldehyde. It is quite apparent under the conditions of the experiment that acetaldehyde did not increase the production of acetylmethylcarbinol.

Results comparable to those above were obtained from Aerobacter indologenes (23 B) and from Aerobacter aerogenes (2 A 4).

Fixation of Heavy Carbon Acetaldehyde in Acetylmethylcarbinol by Aerobacter aerogenes Juice

Preparation of the juice

The cells for this experiment were harvested from a fifteen hour broth culture of Aerobacter aerogenes (2 A4) grown on the glucose, peptone, phosphate medium described previously. Forty-one grams of wet paste were mixed with 82 grams of powdered glass and 0.5 ml. 1M phosphate buffer pH 5.6. The mixture was ground between conical glass cones and collected in 60 ml. cold phosphate buffer (0.5M pH 5.6). The mixture was extracted for thirty minutes and the glass removed by centrifugation in the Swedish angle centrifuge. The supernatant was divided and one portion centrifuged in the "Beams" for six minutes under 80 pounds pressure. This preparation was labeled juice 2. The other portion was centrifuged for ten minutes and labeled juice 2a.

Activity of the preparations

Table 2 shows the relative activities of the two preparations as evidenced by CO_2-production on pyruvate and by formation of acetmethylcarbinol. Each Warburg cup contained 0.3 ml. 0.2 M phosphate buffer, 0.8 ml. sodium pyruvate 0.245M, 0.5 ml. of the juice diluted 1:3 and 0.4 ml. distilled water.

The preparations (Table 2) were very active in the production of acetmethylcarbinol and CO_2. It would appear that the production of CO_2 was increased by the longer centrifugation on the Beams centrifuge. This may be partially explained by a decrease in the endogenous CO_2 caused by the continued centrifugation.

The yields of acetmethylcarbinol were apparently the same in both preparations.

The effect of acetaldehyde on the formation of CO_2 and acetylmethylcarbinol by juice 2a was determined. Each Warburg cup contained the same reagents as in the activity experiment (Table 2) except one cup which contained 0.4 ml. (0.538 M) acetaldehyde and no added distilled water. There was a decrease in the amount of CO_2 formed (Table 3) when the acetaldehyde was added. The inhibition (Graph 2) is manifest in the very early part of the fermentation. The slopes of the curves for both fermentations between 6 and 24 minutes are the same. There is, however, a more rapid leveling off of the curve in the case of the added aldehyde than in the pyruvate fermentation alone. The total

Table 2.

CO_2-Production from Pyruvic Acid by Juices

Prepared from Aerobacter aerogenes

Minutes	CO_2 (mm^3) liberated from pyruvic acid	
	Juice #2	Juice #2a
3	182	258
6	412	589
9	714	1000
13	1044	1425
16	1343	1796
19	1633	2134
23	1944	2453
27	2153	2652
35	2443	2822
61	2582	2994
283	2685	3360
Acetylmethyl-carbinol	+++	+++

Contents of Warburg cups
Phosphate buffer 0.2M pH 6.8 0.3 ml.
Sodium pyruvate 0.245M - 0.8 ml.
Bacterial juice (dil 1:3) 0.5 ml.
Distilled H_2O 0.4 ml.

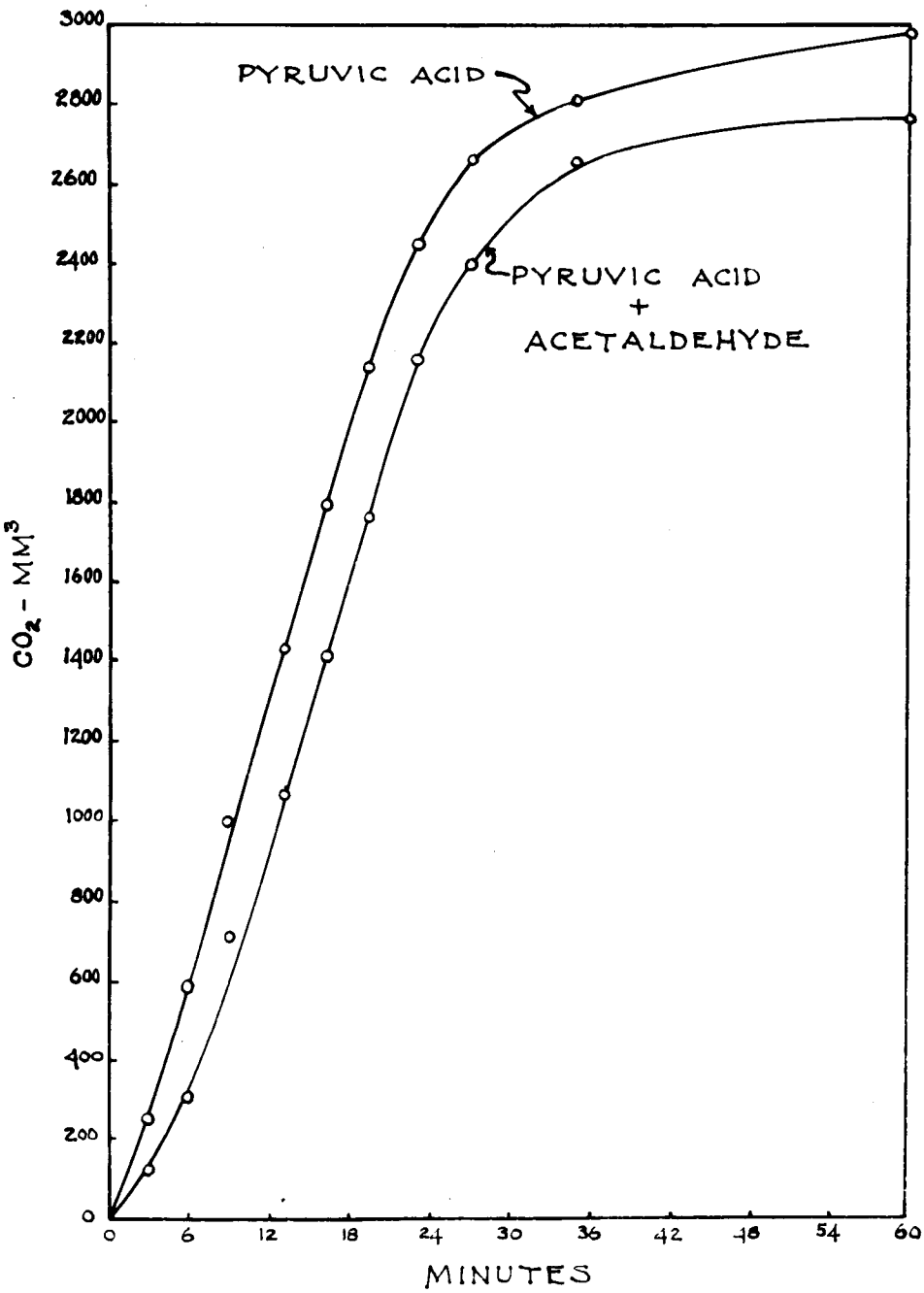
Table 3.

Effect of Acetaldehyde on CO_2-Production from Pyruvic Acid by *Aerobacter aerogenes*

Minutes	CO_2 (mm^3) liberated by juice 2a	
	Pyruvic acid	Pyruvic acid plus Acetaldehyde
3	258	122
6	589	317
9	1000	716
13	1425	1065
16	1796	1419
19	2134	1765
23	2453	2146
27	2652	2387
35	2822	2661
61	2994	2769
283	3360	2889
Acetylmethyl-carbinol	+++	+

GRAPH # 2

EFFECT OF ACETALDEHYDE ON CO_2 PRODUCTION BY AEROBACTER AEROGENES (JUICE 2A) FROM PYRUVIC ACID



amount of CO_2 formed in the pyruvate plus aldehyde fermentation never reached that of the pyruvate fermentation. In this respect the bacterial juice differs from the bacterial suspensions of Mickelson and Werkman (1938, 1939) and of Green (1942).

Less acetylmethylcarbinol was formed in the presence of the aldehyde (Table 3).

Fermentation apparatus

The fermentation apparatus (Diagram 2) consisted of a fermentation chamber and three bead towers. The chamber consisted of a larger pyrex tube stoppered with a three-hole rubber stopper. A piece of glass tubing with a constricted orifice which dipped into the medium was placed in one of these holes. The purpose of this tube was to introduce the O_2-free and CO_2-free nitrogen. In the second hole was placed a small water cooled reflux condenser. In the third hole was a small piece of glass tubing fitted with a piece of rubber tubing and a stopcock for the introduction of the reagents while keeping the system under anaerobic conditions. Bead towers, in series, were connected to the top of the reflux condenser. The first tower contained sodium bisulfite (2%) to trap the aldehyde; the second tower contained $KMnO_4$ ($\frac{1}{2}$ sat.) to trap any SO_2 coming from the bisulfite. The third tower contained 1.5 N CO_2-free NaOH and the whole system was connected to a water-suction pump through a mercury trap. Since a slightly reduced pressure was

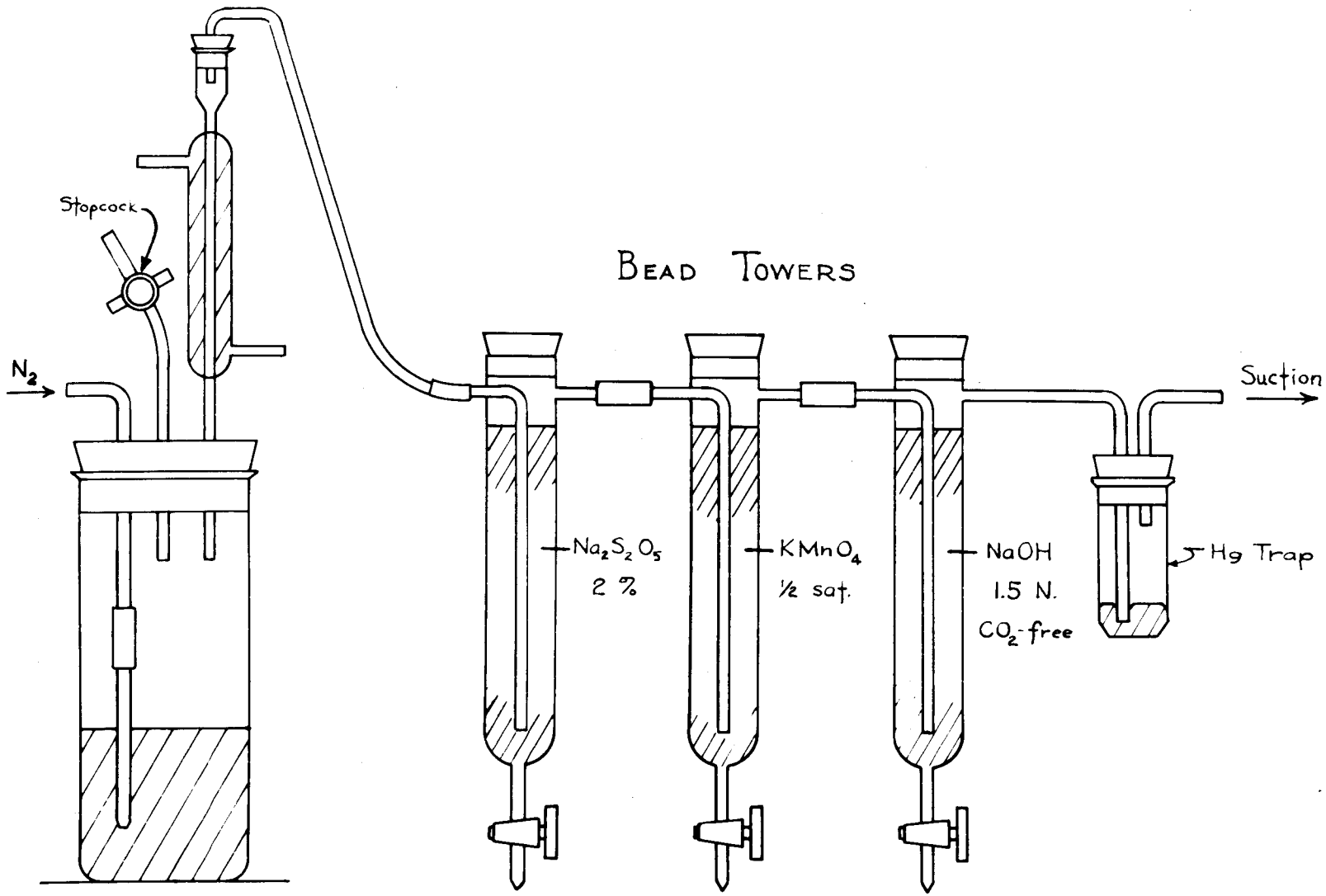


DIAGRAM 2. FERMENTATION APPARATUS

applied to the system, hot paraffin was applied to all stoppers and rubber connections.

Procedure

The fermentation mixture consisted of:

Phosphate buffer	1M pH 5.4	4.5 ml.
Na pyruvate		0.3306 grams (3.00 mM)
Juice 2a		2.5 ml.
Acetaldehyde (C^{13} in both carbons)		22.0 ml. (2.27 mM)
H_2O		1.0 ml.

The pyruvate and the phosphate buffer were first placed in the fermentation chamber. The rubber tubing connecting the reflux condenser to the first bead tower was clamped. CO_2-free, O_2-free nitrogen was then passed through the liquid in the fermentation chamber for fifteen minutes. The clamp was then opened and the nitrogen was passed through the whole system for another fifteen minutes. The nitrogen was then turned off and the other reagents were added. The fermentation was allowed to continue for three and one-half hours.

After incubation 4 ml. 6N H_2SO_4 were added to precipitate the proteins and the fermentation mixture was slowly brought to a boil. The entire system was then swept with nitrogen for 30 minutes to carry over the CO_2 and the acetaldehyde.

The fermentation mixture was made up to 50 ml. and filtered. Aliquots were used (1) to test for residual pyruvate, (2) to test for total carbon, and (3) for the isolation and purification of the acetylmethylcarbinol.

For the isolation and purification of the acetylmethylcarbinol 40 ml. of the fermentation liquor were made acid to congo red and distilled to 20 ml., then steam distilled and 200 ml. collected. Thirty-five millimeters of the distillate were oxidized with $FeCl_3$, according to the method of Stahly and Werkman (1936).

The nickel salt was dissolved with 6 N H_2SO_4 and oxidized with persulfate (Osburn and Werkman, 1932), to obtain the CO_2 for the mass spectrometer sample. The CO_2 was collected in 1.5 N CO_2-free NaOH, and finally precipitated with saturated $BaCl_2$. The precipitate was washed three times in CO_2-free water and dried.

Table 4 shows the results of the mass spectrometer analysis. It must be remembered that naturally occurring carbon compounds contain 1.09 ± 0.02 per cent heavy carbon; thus any product containing more than that amount must contain heavy carbon from the enriched heavy carbon aldehyde.

There were 2.27 mM of acetaldehyde added to the fermentation and 2.14 mM recovered, a loss of 0.13 millimoles. Of the 3 mM of sodium pyruvate added to the fermentation, 2.45 mM were dissimilated. There were 1.04 mM of acetylmethylcarbinol formed. The recovery of the acetaldehyde in the bisulfite solution would indicate that acetaldehyde was not an intermediate in the formation of acetylmethylcarbinol.

It will be observed from Table 4 that the C^{13} content of the original acetaldehyde was 4.25 and that the recovered acetaldehyde was

Table 4.

Addition of Heavy Carbon Acetaldehyde to a

Pyruvic Acid Fermentation of a Juice of Aerobacter aerogenes

Compound	Millimoles	C^{13} per cent
Pyruvate fermented	2.45	-
Acetaldehyde added	2.27	4.25
Acetaldehyde recovered	2.14	4.13
Carbon dioxide liberated	-	1.08
Acetylmethylcarbinol	1.04	1.11
Fermentation liquor	-	1.12

4.13 which may well be within experimental error. The carbon dioxide was 1.08, the fermentation liquor was 1.12 and the acetylmethylcarbinol was 1.11. These values are normal.

Summary and conclusions

The observations of Silverman and Werkman (1941) have been confirmed. The cell-free juice of Aerobacter aerogenes does not utilize added acetaldehyde in forming acetylmethylcarbinol from pyruvate. The heavy carbon acetaldehyde added to the fermentation was all recovered in the bisulfite solution.

Fixation of Heavy Carbon Acetaldehyde in Acetylmethylcarbinol by Yeast Juice

Preparation of the juice

In selecting the dried yeast preparation from which to prepare juices, a preliminary juice was made from each of two old dried yeast preparations. One preparation was made from baker's yeast and air dried by workers in this laboratory. This preparation was two years old and had been stored in the refrigerator. The second dried yeast was brewer's yeast prepared about the same time as the former.

Experiments were conducted to determine which preparation would give the most active acetylmethylcarbinol enzyme preparation. Fifty grams of the air dried preparations were rubbed up with 150 ml. M/15 PO_4 buffer (pH 7.2). This was the pH found by Green (1942) to

to be the optimum for production of acetylmethylcarbinol by yeast juices. The mixture was incubated for one hour at 37° C. with occasional stirring. The autolyzed preparation was diluted with 200 ml. of distilled water and centrifuged on the Swedish angle centrifuge for ten minutes. To each 100 ml. of the supernatant liquid were added 38 grams of $(NH_4)_2SO_4$ to precipitate the protein of the enzyme system. The precipitate thus formed was centrifuged and redissolved in 50 ml. of M/15 phosphate buffer (pH 7.2). The precipitation was repeated and the final precipitate was dissolved in 20 ml. of M/10 phosphate buffer pH 7.2.

Activity of the preparations

The activity of the preparations was determined manometrically by measuring the CO_2 produced under anaerobic conditions. When the manometric tests were completed the relative amounts of acetylmethylcarbinol formed were determined by transferring the contents of the Warburg flasks to a test tube and adding creatine and KOH (40%) according to the procedure of O'Meara (1931).

In the preparation of the juice, some of the precipitate was not dissolved in the amount of phosphate buffer recommended. A suspension of this precipitate was made in 20 ml. of phosphate buffer and tested along with the above juices.

The amount of CO_2 (Table 5) produced from pyruvic acid was greater from the brewer's yeast juice than from the baker's yeast juice. Less

Table 5.

Production of CO_2 and Acetylmethylcarbinol by Preparations of Baker's and Brewer's Yeast from Pyruvic Acid and from Pyruvic Acid Plus Acetaldehyde

Time min.	Baker's juice		Brewer's juice		Baker's precipitate	
	Pyruvate (0.012 M)	Pyruvate (0.012 M) + acetaldehyde (0.061 M)	Pyruvate (0.012 M)	Pyruvate (0.012 M) + acetaldehyde (0.061 M)	Pyruvate (0.012 M)	Pyruvate (0.012 M) + acetaldehyde (0.061 M)
	mm^3	mm^3	mm^3	mm^3	mm^3	mm^3
4	106	107	121	151	45	43
27	158	152	207	274	93	112
42	164	152	211	278	100	117
81	163	142	221	275	111	116
A.M.C.	++	++++	+	++	-	-

than half as much CO_2 was produced from the precipitate fraction than from the supernatant. The difference in the activity might have been due to more precipitate being dissolved in the first 20 ml. of phosphate buffer. No attempt was made to determine the amount of precipitate dissolving in either fraction.

The addition of acetaldehyde inhibited slightly the production of CO_2 in the case of the baker's yeast and enhanced it for the brewer's yeast.

The amount of acetylmethylcarbinol formed was much greater for the baker's preparation than for the brewer's yeast. It was of interest to note the amount of acetylmethylcarbinol was about doubled with both yeast preparations when acetaldehyde was added. There was no carbinol formed by the precipitate suspension of the baker's yeast.

As a result of these data, the baker's yeast was used in preparing the juices used in the following experiments.

Effect of dilution on the enzyme preparation

Since the yeast preparation of Green (1942) was employed in much greater concentration than the bacterial juices of Silverman and Werkman (1941), the effect of dilution on the activity of the yeast enzyme preparation was determined.

The enzyme was a part of the baker's preparation described above. The cup contents were the same as above but the amounts of the preparation varied from 0.37 ml. to 1.5 ml.

The total CO_2 produced (Table 6) when 0.37 ml. of the juice was used was 235 mm^3. This amount of CO_2 was about half that produced when 1.5 ml. of juice was employed. The greater the concentration of juice, the larger the amount of CO_2 produced. Time was not a factor because in all three fermentations the reactions were about complete after 38 minutes. Regardless of the concentration of enzyme, the production of CO_2 stopped after 120 minutes.

The amount of acetylmethylcarbinol produced (Table 6) increased with the greater concentration of enzyme. There appeared to be a direct relationship of the amount of CO_2 produced and the amount of acetylmethylcarbinol formed.

Effect of freezing on activity of juice

A portion of baker's juice was frozen in a refrigerator and stored for six days. The preparation was then thawed in a water bath at 30.4° C. and tested manometrically for activity. When the activity of the frozen juice is compared with that of the fresh juice (Graph 3), one will find the rate of CO_2-production a little less in the frozen juice preparation. The total amount of CO_2 is considerably less in the frozen juice fermentation but enough CO_2 is formed to show considerable activity remaining in the juice after freezing.

The acetylmethylcarbinol formed (Table 7) was approximately the same as that produced by the fresh juice.

Table 6.

Effect of Enzyme Dilution on CO_2-production and Acetyl-methylcarbinol-formation

Minutes	Pyruvate (0.012 M) plus Acetaldehyde (0.061 M)		
	Amount of enzyme preparation		
	1.5 ml.	0.75 ml.	0.37 ml.
	mm^3	mm^3	mm^3
8	374	198	81
15	482	285	146
38	552	317	215
120	565	354	235
167	565	354	235
A.M.C.	+++	++	+

GRAPH # 3

EFFECT OF FREEZING ON ACTIVITY OF YEAST JUICE

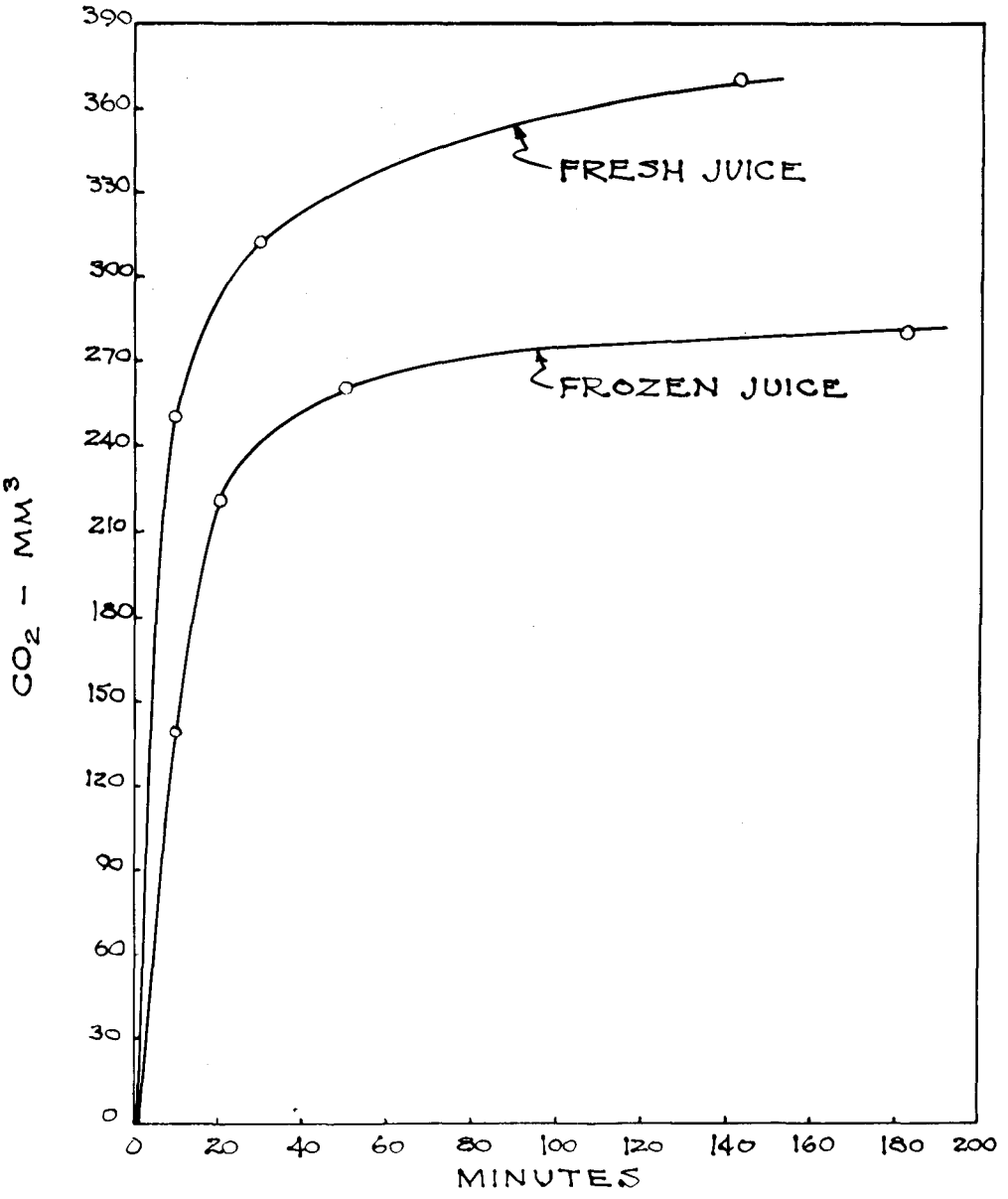


Table 7.

Effect of Freezing Yeast Juice on CO_2-production and Acetylmethylcarbinol Formation

Minutes	Fresh juice pyruvate 0.015 M	Frozen juice pyruvate 0.013 M
10	247	142
22	---	218
30	312	---
50	---	261
144	367	---
182	---	276
A.M.C.	+	+

The effect of the addition of acetaldehyde upon the activity of the frozen enzyme preparation is recorded in Table 8. The rate of CO_2-production from pyruvate plus acetaldehyde by fresh yeast juice and by frozen yeast juice are compared in Graph 4. The rate of CO_2-production is a little less with the frozen juice. The amount of acetylmethylcarbinol formed was the same (Table 8).

The addition of acetaldehyde (Table 9) increases the yield of CO_2 and of acetylmethylcarbinol.

Effect of concentration of substrate on the activity of the juice

Frozen juice was used in these experiments. Two concentrations of pyruvic acid were used, 0.015 M and 0.130 M.

The total amount of CO_2 formed from 0.13 M pyruvate was five times as much as was formed from the 0.015 M solution (Table 10). The rate of CO_2evolution, during the first 10 minutes of the experiment, was considerably greater in the more concentrated pyruvate solution.

The amount of acetylmethylcarbinol formed was about twice as much in the more concentrated solution.

A comparison of results in Tables 10 and 11 shows that the addition of acetaldehyde to the lesser concentration of pyruvate increased the rate of production of CO_2 for 30 minutes but the total amounts of CO_2 in both cases were about the same. In the greater concentration of pyruvate the reverse is the case. The rate of CO_2-production was considerably higher in the cups containing pyruvate alone at the beginning

Table 8.

Effect of Freezing on CO_2-production and Acetylmethylcarbinol Formation from Pyruvate plus Acetaldehyde by Yeast Juice

Minutes	Fresh juice Pyruvate 0.015 M Acetaldehyde 0.061 M	Frozen juice Pyruvate 0.13 M Acetaldehyde 0.047 M
	mm^3	mm^3
10	303	202
22	-	279
30	357	-
50	-	309
144	397	-
182	-	313
A.M.C.	++	++

Graph # 4

EFFECT OF FREEZING
ON CO_2-PRODUCTION AND A.M.C. FORMATION
BY YEAST JUICE
FROM PYRUVATE PLUS ACETALDEHYDE

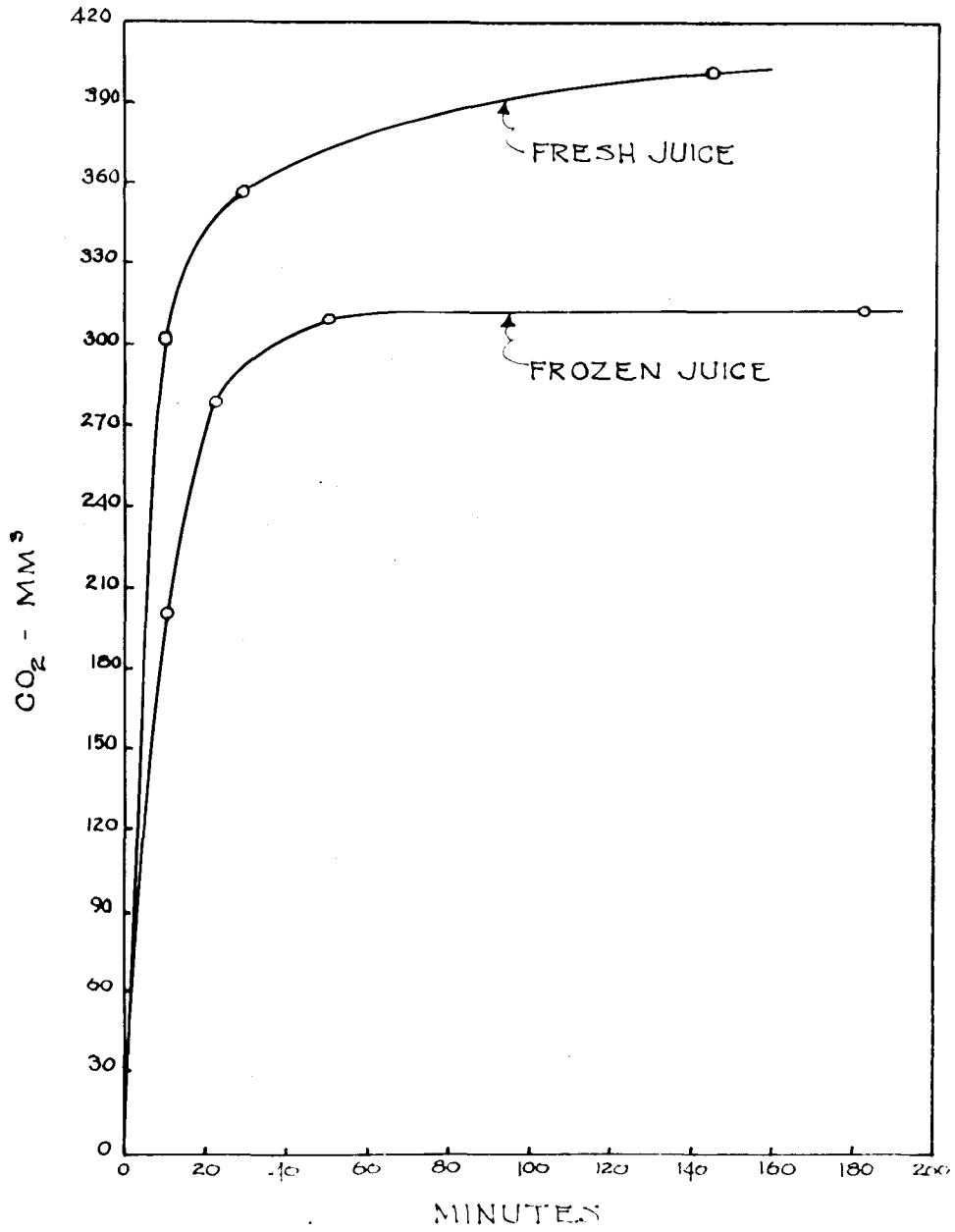


Table 9.

Effect of Acetaldehyde on CO_2-production from Pyruvate by Frozen Yeast Juice

Minutes	Pyruvate* 0.013 M	Pyruvate 0.013 M Acetaldehyde 0.047 M
	mm^3	mm^3
10	142	202
22	218	279
50	261	309
182	276	313
A.M.C.	+	++

Table 10.

Effect of Concentration of Sodium Pyruvate on the CO_2-production and Acetylmethylcarbinol Formation by Frozen Yeast Juice

Minutes	Pyruvate 0.015 M	Pyruvate 0.13 M
	mm^3	mm^3
1	92	378
3	166	784
6	222	1222
10	247	1421
12	274	1561
30	312	1813
51	315	1875
144	367	1864
A.M.C.	+	++

Table 11.

Effect of Concentration of Sodium Pyruvate on the CO_2-production and Acetylmethylcarbinol Formation of Frozen Yeast Juice in the Presence of Acetaldehyde

Minutes	Pyruvate 0.015 M Acetaldehyde 0.06 M	Pyruvate 0.13 M Acetaldehyde 0.093 M
	mm^3	mm^3
1	131	204
3	217	489
6	276	870
10	303	1195
12	324	1474
30	357	2085
51	359	2245
144	397	2492
A.M.C.	++	++++

of the experiment. The total amount of CO_2 produced was considerably higher with the cup containing the acetaldehyde.

Fermentation procedures

The fermentation mixture consisted of:

Phosphate buffer M/2 pH 7.6	1 ml.
Enzyme preparation	15 ml.
Na pyruvate	0.2478 gr. (2.25 mM)
Acetaldehyde (C^{13}).1014 M	15 ml. (1.52 mM)
H_2O (CO_2-free)	3 ml.
Total	34 ml.

The apparatus used for this investigation was the same as the one employed with the bacterial juice.

The enzyme preparation was added to the fermentation tube and the system up to the first bead tower was flushed with O_2-free and CO_2-free nitrogen. The sodium pyruvate* was weighed directly into the acetaldehyde. After anaerobic conditions throughout the system were attained, the other reagents were added. The temperature was kept at 38° C. The fermentation was continued for two hours and twenty minutes when 6 ml. 6N H_2SO_4 were added and the mixture slowly brought to a boil. The water-bath was removed to facilitate heating the fermentation chamber. A slightly reduced pressure was maintained throughout the experiment. After the liquid had come to a boil, nitrogen was gently

*The sodium pyruvate used in this experiment was prepared according to the method of Peters (1938).

bubbled through the fermentation mixture for thirty minutes to remove the acetaldehyde which was collected in 50 ml. of a 2 per cent bisulfite solution and made up to 100 ml. The CO_2 was collected in 10 ml. CO_2-free NaOH (1.5N) solution and made up to 100 ml.

The fermentation mixture was made up to 110 ml. and filtered. A qualitative creatine test revealed the presence of acetylmethylcarbinol.

An aliquot of the fermentation liquor was oxidized by ceric sulfate to determine the amount of unfermented pyruvate present. Another aliquot was oxidized by persulfate to determine the amount of C^{13} present in the fermentation liquor. A third aliquot of 85 ml. was made alkaline to phenolphthalein and concentrated by distillation to 15 ml., then steam distilled and 200 ml. collected. Considerable quantities of NH_3 distilled off under these conditions.

An aliquot of the alkaline distillate was oxidized with $FeCl_3$ and the diacetyl collected as the Ni-glyoxime according to the method of Stahly and Workman (1936).

The samples for the mass spectrometer were prepared in a manner described for the bacterial preparations.

There were 1.52 millimoles of heavy carbon acetaldehyde added to the fermentations in the first experiment and there were 2.28 millimoles of aldehyde recovered (Table 12). These results indicate that acetaldehyde was formed by the fermentation. This increase in acetaldehyde results in a considerable dilution as evidenced by the

Table 12.

Heavy Carbon Acetaldehyde Fixation by Yeast Juices

Compound	First fermentation		Second fermentation	
	mM	Per cent C^{13}	mM	Per cent C^{13}
Original C^{13} acetaldehyde	1.52	4.25	3.27	4.76
Recovered acetaldehyde	2.28	1.97	3.76	3.37
Carbon dioxide	1.82	1.09	2.83	1.07
Fermentation liquor	-	1.41	-	1.39
Acetylmethylcarbinol	0.32	1.97	0.52	2.53
Pyruvate fermented	1.84	-	2.91	-

fact that the original aldehyde contained 4.25 per cent C^{13}, whereas the recovered aldehyde contained 1.97 per cent. There were 0.325 millimoles of acetylmethylcarbinol formed with a C^{13} content of 1.97 per cent. This is considerably above the normal (1.09); therefore the heavy carbon acetaldehyde had to enter into the formation of the acetylmethylcarbinol.

The C^{13} content of the fermentation liquor was 1.41 per cent; thus a considerable amount of acetaldehyde had been transformed into some compound that was not carried over by the stream of nitrogen.

The CO_2 liberated from the fermentation contained no excess C^{13} and therefore did not originate in the acetaldehyde added to the fermentation.

There were 1.84 millimoles of sodium pyruvate fermented by the yeast juice. It will be observed that for each millimole of pyruvate fermented there was one millimole of CO_2 formed.

In the second experiment 3.27 millimoles of heavy carbon acetaldehyde were used and 3.76 millimoles of acetaldehyde were recovered, an increase of 0.49 millimoles. This increase was not as large as in the first experiment but it does indicate the formation of acetaldehyde from the pyruvic acid. This biologically formed acetaldehyde appreciably diluted the heavy carbon aldehyde as evidenced by the per cent of C^{13} determined. The original aldehyde contained 4.76 per cent C^{13}, whereas the recovered aldehyde contained 3.37 per cent.

There was approximately one millimole of CO_2 formed for each millimole of pyruvate fermented in both experiments. The C^{13} of the CO_2 was normal (1.09 per cent).

It will be observed that the increase in C^{13} content of the fermentation liquor was approximately the same. In the first experiment the C^{13} content was 1.41 while in the second experiment it was 1.39.

The ratio of acetylmethylcarbinol formed to pyruvate fermented was in the former experiment 0.173 and in the latter experiment 0.178, which is approximately the same, but the C^{13} content of the two preparations were not the same. It is true the C^{13} contents of the two original aldehydes were not the same but the difference is not proportional. The ratio of original aldehyde to acetylmethylcarbinol in the former experiment was 2.26 while that for the latter experiment was 1.88. This indicates a greater proportion of C^{13} carbon atoms in the acetylmethylcarbinol in the latter experiment than in the carbinol of the former experiment.

Summary and conclusions

The yeast juice preparation of Green (1942) produced acetylmethylcarbinol containing increased amounts of heavy carbon. The only source of this heavy carbon was the added acetaldehyde. The juice preparations utilized synthetic heavy carbon acetaldehyde, confirming the early work of Neuberg and also that of Green (1942). The aldehyde formed from the pyruvate had a considerable dilution effect on the heavy carbon

acetaldehyde. The CO_2 formed must have been from the pyruvate, as it could not have come from the acetaldehyde with the C^{13} content remaining normal. The evidence here does not indicate a definite ratio of one synthetic aldehyde to one biologically formed aldehyde as suggested by Neuberg.

Formation of Acetylmethylcarbinol by Pig Heart Juice

Preparation of the juice

The enzyme preparation was made according to the method of Green (1942). The hearts (236 grams), just removed from three pigs, were minced twice in a coarse meat grinder. This mince was washed five times with ten volumes of tap water. To each portion of eighty grams of pressed mince, there were added forty milliliters of M/2 β-glycerophosphate buffer (pH 6.0) and 240 grams of crushed ice. This mixture was homogenized for ten minutes in a Waring blender.

The suspension was centrifuged in a Swedish angle centrifuge. The cloudy supernatant was decanted and mixed with 0.5 volume of crushed ice. The supernatants of all the homogenized portions were mixed together. The enzyme was precipitated by cautiously adjusting the mixture to pH 4.6 with acetic acid (10 per cent). The precipitate was quickly removed by centrifugation in the cold. The precipitate was resuspended in a mixture of 25 ml. of M/2 phosphate buffer pH 6.0 and 5 ml. of M/2 $NaHCO_3$.

Activity of enzyme preparation

The activity of the preparation was determined manometrically. Each cup on the Barcroft-Warburg respirometer contained one milliliter of the enzyme preparation, 0.1 ml. diphosphothiamin (150 γ/ml.), 0.1 ml. $MgSO_4$ (0.1%), substrate and enough water to make a total volume of 2 ml. The substrate was placed in the side arm of the respirometer cup. After the other reagents were added the cups were made anaerobic by flushing with nitrogen. The cups were then placed in the water bath and shaken until temperature equilibrium had been established. The contents of the side arm were dumped into the main vessel and the amount of CO_2 produced recorded.

It will be observed from Table 13 that from pyruvate there were 16 mm^3 of CO_2 evolved in the first ten minutes; there were 38 mm^3 of CO_2 produced from pyruvate plus acetaldehyde during the same period. It would appear that the presence of acetaldehyde increased CO_2-production. The total amount of CO_2 formed from pyruvate plus acetaldehyde, however, was considerably less than from the pyruvate alone.

The total amount of CO_2 produced by pig heart juice was much less than that from either the bacterial or the yeast juice.

There was no CO_2 formed from the acetaldehyde during the entire experiment.

It will be observed (Table 13) that the addition of acetaldehyde to pyruvate increased greatly the amount of the acetylmethylcarbinol;

Table 13.

CO_2-Production from Pyruvate, Acetaldehyde and Pyruvate plus Acetaldehyde by a Juice from Pig Heart

Minutes	Pyruvate .03 M	Acetaldehyde .036 M	Pyruvate .03 M plus acetaldehyde .036 M
	mm^3	mm^3	mm^3
10	16	0	38
165	60	0	42
280	66	-2	40
A.M.C.	++	++++	++++

however, there was as much carbinol formed from the acetaldehyde alone as from the acetaldehyde plus pyruvate. The total amount of acetylmethylcarbinol, as evidenced by the O'Meara creatine test, was much greater than that formed by either the bacterial or the yeast juices. This juice has shown the ability to produce large amounts of acetylmethylcarbinol without forming large amounts of CO_2 and from acetaldehyde without producing any CO_2.

Summary and conclusions

A pig heart juice was prepared which produced acetylmethylcarbinol from either pyruvate or acetaldehyde. The presence of acetaldehyde increased the production of CO_2 from pyruvate during the first ten minutes of the experiment. These observations are in agreement with Green et al. (1942).

It appears that the early work with yeast establishing a ratio of CO_2 to acetylmethylcarbinol of 1 does not hold for the pig heart juice, as large amounts of the carbinol were formed from acetaldehyde without any CO_2 being formed.

Degradation of Heavy Carbon Acetylmethylcarbinol Formed by Yeast Juice

Purification of heavy carbon acetylmethylcarbinol

The acetylmethylcarbinol formed in the fermentation process by the yeast juice was retained in the fermentation liquor and thus had

to be purified before degradation could be conducted successfully. First attempts to remove the acetylmethylcarbinol by alkaline distillation followed by steam distillation were not satisfactory because of the large amounts of ammonia passing over. Only a small amount of acetylmethylcarbinol could be recovered in the distillate. When the fermentation liquor was made acid to congo red before steam distilled, the recovery of the acetylmethylcarbinol was satisfactory. The acid distillate was placed in a distillation flask and distilled until the volume in the flask was 20 ml. Steam was passed into the flask until 200 ml. were collected. To remove the volatile acids carried over, the distillate was made alkaline to phenolphthalein and distilled until the volume in the distillation flask was 20 ml., steam distilled, until a total of 400 milliliters of distillate were collected. This alkaline distillate was refluxed for two hours to remove certain sulfur compounds present in the steam.

Degradation procedures

The methyl group next to the keto carbon was split off by the iodoform reaction following the method of Langlykke and Peterson (1937). To an aliquot of the alkaline distillate were added CO_2-free NaOH and iodine solution. The alkali was added first. The iodine was added slowly drop by drop with constant shaking of the flask. The reaction was allowed to continue for fifteen minutes when just enough

acid (H_2SO_4) was added to neutralize the sodium hydroxide. Then 0.1 ml. of 6 N H_2SO_4 excess was added to acidify the mixture. The excess iodine was titrated with sodium thiosulfate. No starch was added as an indicator; therefore the titration end point was not a sharp one. The iodoform was removed by filtration through a sintered glass crucible, washed and then dried for two days in a desiccator over $CaCl_2$. The dried iodoform was weighed and calculated. It was treated with 6 N H_2SO_4 and oxidized by the Friedemann and Kendall (1929) method. The CO_2 was collected in CO_2-free NaOH.

In later experiments the iodoform was collected on asbestos pads, which when dry could be oxidized directly without the H_2SO_4 step as considerable amounts of iodoform escaped during this process.

The filtrate from the iodoform filtration, containing the lactic acid, was treated with $AgNO_3$ (10 per cent) to remove the excess HI. The precipitate was removed by filtration and the Ag^+ was removed with HCl and the precipitate again removed by filtration.

Preliminary experiments showed that the iodine interfered in the oxidation of the lactic acid, and thus had to be removed in the above manner.

The lactic acid was oxidized by a modification of the Friedemann and Graeser (1933) method. The solution containing the lactic acid was placed in a 300 ml. Erlenmeyer flask, and 10 ml. $MnSO_4$ were added. The flask was connected to a water cooled reflux condenser which was attached to a bead tower containing sodium bisulfite. CO_2-free air

was slowly passed through while $KMnO_4$ was added drop by drop until a brown color appeared. The CO_2 was collected in a bulb containing CO_2-free NaOH. Between the sodium bisulfite tower and the NaOH tower was a bead tower containing saturated $KMnO_4$ to collect SO_2 passing over from the bisulfite.

In later experiments the lactic acid was purified by making the solution acid to congo red and evaporating to 25 ml. This was then steam distilled and 250 ml. of distillate collected. The residue was made alkaline and was ether extracted continuously for twelve hours. The ether fraction was made acid and again extracted for eleven hours. The ether was distilled off and the solution evaporated to half volume. $AgNO_3$ was added and the mixture filtered. The Ag^+ was removed with HCl. The $MnSO_4$ was added and the mixture was refluxed with CO_2-free air for fifteen minutes. The $KMnO_4$ was then added and the procedure followed as before.

The acetaldehyde obtained from the oxidation of the lactic oxidation was degraded according to the iodoform reaction.

The formic acid, in the filtrate from the iodoform reaction, was made acid to congo red and placed in a 300 ml. distillation flask. The volume was distilled to 25 ml. and then steam distilled, a total volume of 500 milliliters was collected. The distillate was refluxed for two hours and neutralized to phenolphthalein. A slight excess NaOH was added and the solution was boiled down to 200 milliliters. The

whole was placed in a distillation flask and distilled to fifteen milliliters. The contents were cooled and acidified with H_2SO_4 to congo red. The acidified solution was steam distilled and 200 milliliters collected. The distillate was refluxed for fifteen minutes. This refluxed solution was cooled and two grams of HgO were added and the flask was attached to the CO_2 outfit described above. The mixture was refluxed for twenty minutes while CO_2-free air was passed through. Five milliliters of 25 per cent phosphoric acid were added and aeration and boiling were continued for another fifteen minutes. The CO_2 formed was collected in CO_2-free NaOH.

In still other degradation experiments the acetylmethylcarbinol was cleaved by the periodate oxidation to acetaldehyde and acetic acid. An aliquot of the purified acetylmethylcarbinol was oxidized with KIO_4 according to the method of Stahly and Werkman (1936), and the aldehyde collected in sodium bisulfite.

The aldehyde obtained was oxidized by persulfate to CO_2 which was collected in CO_2-free NaOH.

The residue obtained from the KIO_4 oxidation was made acid to congo red and distilled to fifteen milliliters. It was then steam distilled and 500 ml. collected. The distillate was then refluxed for two hours. The solution was made alkaline to phenolphthalein and evaporated to 10 ml. This was made acid to congo red and steam distilled to 100 ml. and the distillate refluxed for fifteen minutes. The refluxed distillate was neutralized and evaporated to dryness. The residue was taken up in CO_2-free water and filtered. This solution

was oxidized by persulfate to CO_2 which was collected in CO_2-free NaOH.

In all the above cases the CO_2 from the oxidation procedures was liberated from the sodium hydroxide with 4 N lactic acid into a previously evacuated extraction bulb containing anhydrone to remove the water. The CO_2 was then analyzed on the mass spectrometer for C^{13}.

Results of the degradation of heavy carbon acetylmethylcarbinol

The results of the first degradation of the heavy carbon acetylmethylcarbinol formed by a yeast juice preparation are recorded in Table 14. The amount of acetylmethylcarbinol used was 0.17 millimoles and had a C^{13} content of 1.97. The iodoform, coming from the acetyl group (0.11 millimoles), contained only 1.19 per cent C^{13}. Preliminary experiments with ordinary acetylmethylcarbinol always yielded low yields of iodoform. This compound is so volatile that the amount missing could well have been lost by evaporation in the drying procedure; however, the lactic acid formed as determined by the Friedemann and Graeser method was only 0.12 millimoles. The iodoform formed from the acetaldehyde obtained from the lactic acid determination contains the carbon of the methyl group next to the carbinol carbon. There was 0.10 millimole of iodoform recovered which contained 1.36 per cent heavy carbon. The amount of CO_2 from the lactic acid was not determined but the C^{13} content was 1.35 per cent. The formic acid obtained from the degradation of the acetaldehyde from the lactic acid contained 1.83 per cent heavy carbon.

Table 14.

Degradation of Heavy Carbon Acetylmethylcarbinol Produced from Heavy Carbon Acetaldehyde by Yeast Juice Preparation

Compound	Part of A.M.C.	Millimoles	Per cent C^{13}
Acetylmethylcarbinol (Ni salt)	whole molecule	0.17	1.97
Iodoform	CH_3 of $CH_3-\overset{O}{\overset{\|}{C}}-$	0.11	1.19
Lactic acid formed	$-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}(\overset{H}{\overset{\|}{O}}) - CH_3$	0.12	-
CO_2 from lactic acid	$-\overset{O}{\overset{\|}{C}}-$	-	1.35
Iodoform from acetaldehyde from lactic acid	CH_3 of $CH_3-\overset{O}{\overset{\|}{C}}(\overset{H}{\overset{\|}{O}})-$	0.10	1.36
Formic acid from acetaldehyde from lactic acid	$-\overset{O}{\overset{\|}{C}}(\overset{H}{\overset{\|}{O}})-$ of $CH_3-\overset{O}{\overset{\|}{C}}(\overset{H}{\overset{\|}{O}})-$	-	1.83
Acetaldehyde from KIO_4 oxidation of A.M.C.	$CH_3-\overset{O}{\overset{\|}{C}}(\overset{H}{\overset{\|}{O}})-$	0.02	1.43

The original acetylmethylcarbinol contained 1.97 per cent heavy carbon. The CO_2 (carbonyl carbon) and the iodoform (CH_3 next to the carbinol group) contained 1.35 and 1.36 per cent heavy carbon respectively. The formic acid (carbinol carbon) contained 1.83 per cent heavy carbon which is closest to the per cent heavy carbon in the original acetylmethylcarbinol molecule.

There is no doubt that heavy carbon from the heavy carbon aldehyde has gone into all carbons in the acetylmethylcarbinol. If one assumes that the acetylmethylcarbinol was formed from one biologically active acetaldehyde and one synthetic acetaldehyde, one-half of the acetylmethylcarbinol would contain the increased concentration of heavy carbon, provided of course that acetylmethylcarbinol was not reduced to 2,3-butylene glycol, (or oxidized to diacetyl) and oxidized (or reduced) again to acetylmethylcarbinol. Thus if acetylmethylcarbinol was oxidized with KIO_4 and the molecule split to acetaldehyde and acetic acid, one compound should contain the increased heavy carbon. Eight ml. of the fermentation liquor, containing 0.02 millimoles of acetylmethylcarbinol, were oxidized to CO_2 by persulfate and the heavy carbon determined. The amount of heavy carbon (1.43 per cent) contained in the acetaldehyde derived from the carbinol fraction of the acetylmethylcarbinol was only slightly higher than that obtained in previous degradation procedures.

One suggested explanation for the decrease in heavy carbon involves CO_2-dilution from the procedures used in degrading the compounds. It

will be observed from Table 14 that a relatively small amount of acetylmethylcarbinol was used. All experimental procedures were rechecked to determine whether there were any dilution effects present in degrading ordinary acetylmethylcarbinol. Only a slight effect could be detected.

An experiment was designed to minimize the small CO_2-dilution effect and to determine the C^{13} content of each half of the acetylmethylcarbinol molecule. The remaining alkaline filtrates, containing the heavy carbon acetylmethylcarbinol, were oxidized by periodate. Sixty-five hundredths of a millimole of C^{13} acetylmethylcarbinol were employed. The amounts of C^{13} in the compounds are recorded in Table 15. The whole acetylmethylcarbinol molecule contained 2.14 per cent C^{13}. The acetaldehyde, representing the carbinol end of the molecule, contained 1.95 per cent C^{13} and the acetic acid, representing the carbonyl end of the molecule, contained 2.03 per cent C^{13}.

These data indicate that C^{13} is evenly distributed among the carbon atoms of the molecule.

A larger amount of heavy carbon acetylmethylcarbinol was degraded by the same procedures used in the first degradation. It will be observed (Table 16) that 0.53 millimole of acetylmethylcarbinol was employed, and 0.45 millimole of iodoform obtained from the addition of iodine to the alkaline distillate of the carbinol. There was 0.46 millimole of acetaldehyde and 0.60 millimole of CO_2 from the oxidation of the lactic acid formed in the iodoform reaction. An aliquot of

Table 15.

Degradation of Acetylmethylcarbinol by Periodate Oxidation

Compound	Part of A.M.C.	Millimoles	Per cent C^{13}
A.M.C.	whole	.65	2.14
Acetaldehyde	$CH_3-\overset{H}{\underset{I}{C}}-OH$	.62	1.95
Acetic acid	$CH_3-\overset{}{\underset{I}{C}}=O$	-	2.03

Table 16.

Degradation of Heavy Carbon

Acetylmethylcarbinol

Compound	Part of the A.M.C. molecule	Millimoles	Per cent C^{13}
Acetylmethylcarbinol	whole	0.53	2.53
Iodoform	CH_3 of $CH_3-C=O$	0.45	2.35
Aldehyde from lactic acid	$CH-\overset{H}{C}OH$	0.46	3.11
CO_2 from Lactic acid	$\overset{O}{\overset{\|}{C}}$	0.60	1.53
Aldehyde from lactic acid (liberated from bisulfite before persulfate oxidation)	$CH_3-\overset{H}{C}-OH$	-	3.16

aldehyde was oxidized directly from the bisulfite by persulfate. Another aliquot was liberated from the bisulfite before oxidation.

The C^{13} content of the whole acetylmethylcarbinol molecule was 2.53 per cent; the iodoform 2.35 per cent and the CO_2 from the lactic acid was 1.53 per cent. The aldehyde from the lactic acid was 3.11 per cent C^{13} from the sample oxidized directly from the bisulfite, and 3.16 per cent from the sample liberated from the bisulfite before the oxidation. These data indicate the heavy carbon contents of the various carbons are different. The carbonyl fraction of the molecule contains the smaller amount of heavy carbon. The carbinol fraction contains more than the whole molecule indicating a greater fixation of the synthetic acetaldehyde in the carbinol portion.

Acetylmethylcarbinol Formation from Various Yeast Preparations

During the course of this investigation the dried yeast preparations on hand became exhausted and new sources were sought. It was reported by Silverman (1941) that of the large number of active yeast cultures tested only a very few produced acetylmethylcarbinol. Many of these yeasts after being air dried produced considerable amounts of the carbinol.

Juices prepared from many dried preparations of both baker's and brewer's yeasts formed only traces of acetylmethylcarbinol. Juices

from many of these preparations, however, produced considerable amounts of CO_2 from pyruvate and from pyruvate plus acetaldehyde.

Many commercially dried yeast preparations were also used but no good acetylmethylcarbinol-forming juice was obtained.

One preparation made from baker's yeast was incubated at 37° C. after being air dried. This preparation showed promise. There was a considerable increase in acetylmethylcarbinol formation as the preparation was incubated.

DISCUSSION

This investigation has contributed to the knowledge of the mechanism of formation of acetylmethylcarbinol by clarifying the observations of Silverman and Werkman (1941) and Green *et al.* (1942).

Silverman and Werkman (1941) prepared a bacterial juice from *Aerobacter* that formed acetylmethylcarbinol from pyruvate without apparent utilization of acetaldehyde. Green *et al.* (1942) prepared a juice from dried brewer's yeast that formed acetylmethylcarbinol from pyruvate alone but the amount of acetylmethylcarbinol was greatly increased when acetaldehyde was added. The rate of evolution of CO_2 during the first thirty minutes of the fermentation was greatly increased when acetaldehyde was added.

It appeared that the results of these investigators were not in agreement as to the role of acetaldehyde.

This investigation has shown that the bacterial juice of Silverman and Werkman does not utilize synthetic acetaldehyde in the formation of acetylmethylcarbinol. Heavy carbon acetaldehyde, with C^{13} in both positions, when added to a pyruvic acid fermentation yielded acetylmethylcarbinol with only the normal percentage of heavy carbon. The heavy carbon acetaldehyde added amounted to 2.27 millimoles and 2.14 millimoles were recovered in the bisulfite. The heavy carbon of the added aldehyde amounted to 4.25 per cent and that of the recovered

aldehyde was 4.13. If acetaldehyde had been formed during the fermentation, there would have been an increase in the amount of acetaldehyde recovered with a subsequent drop in the per cent of heavy carbon. On the other hand, if acetaldehyde had been formed from the pyruvate, the juice could utilize only the biologically formed acetaldehyde. All of the biologically formed acetaldehyde was utilized and only the synthetic was carried over into the bisulfite solution. This is evidenced by the fact that, within experimental error, only the added acetaldehyde was recovered.

This bit of evidence would suggest that possibly some other compound was serving as an intermediate. Lipmann (1939) indicated that in the breakdown of pyruvate by Lactobacillus delbrückii a phosphorylated intermediate was formed. It may be possible that some such phosphorylated compound is functioning here.

The yeast juice enzyme preparation of Green et al.,on the other hand, can utilize synthetic acetaldehyde in the formation of acetylmethylcarbinol. When heavy carbon enriched acetaldehyde was added to yeast juice fermentations of pyruvate, acetylmethylcarbinol was formed containing increased amounts of heavy carbon.

In one experiment 3.27 millimoles of synthetic heavy carbon aldehyde were added; 3.76 millimoles were recovered in the bisulfite solution. The heavy carbon content of the original aldehyde was 4.76 per cent, whereas that of the recovered aldehyde was 3.37 per cent. The increase in the amount of acetaldehyde recovered and the decrease

in its heavy carbon content indicate that acetaldehyde was formed during the fermentation.

There was 0.52 millimole of acetylmethylcarbinol formed containing 2.53 per cent heavy carbon. The heavy carbon content of the acetylmethylcarbinol is approximately half that of the added heavy carbon acetaldehyde. These data indicate that one molecule of synthetic aldehyde combined with one molecule of biologically formed aldehyde to form one molecule of acetylmethylcarbinol. This finding would support the early suggestions of Neuberg.

The CO_2 formed must have come from the decarboxylation of the pyruvate because there was no increase in the heavy carbon content.

These investigations, therefore, confirm the observations of Green et al. (1942).

Green et al. prepared a juice from pig heart which appears to be quite different from either the bacterial juice of Silverman and Werkman or the yeast juice of Green. This preparation formed acetylmethylcarbinol from acetaldehyde alone. Green found that diphosphothiamine was essential.

It was found in these investigations that fresh pig heart enzyme preparation formed large quantities of acetylmethylcarbinol from acetaldehyde without the production of CO_2. There were 16 microliters of CO_2 formed from pyruvate and 38 microliters from pyruvate plus acetaldehyde in the first ten minutes of the fermentation. This would indicate a stimulatory effect of adding the acetaldehyde.

The total CO_2 evolved, however, was 60 microliters for the pyruvate and 40 microliters for the pyruvate plus acetaldehyde. The amount of acetylmethylcarbinol produced from pyruvate plus acetaldehyde was about twice that from pyruvate alone, but that from the adetaldhyde alone was equal to that produced from pyruvate plus acetaldehyde.

The answer to the problem of the formation of acetylmethylcarbinol from the synthetic and biological acetaldehyde is not clear. Neuberg et al. believed that an enzyme carboligase condensed one biologically formed molecule of aldehyde with one synthetic aldehyde. If this were true, in the acetylmethylcarbinol formed by the yeast juice heavy carbon should appear in only one-half of the molecule. This statement assumes that acetylmethylcarbinol is not oxidized or reduced to a symmetrical molecule which can be converted to acetylmethylcarbinol.

The heavy carbon acetylmethylcarbinol was degraded in order to study this problem. It was found that all carbon atoms contained an increase in heavy carbon. In some experiments the carbinol carbon had the greatest concentration of heavy carbon. Splitting the acetylmethylcarbinol by periodic oxidation, however, gave equal concentrations of heavy carbon in both fractions.

These data indicate that there is no set rule of one biologically formed acetaldehyde joining with one synthetic acetaldehyde molecule if the formation of a symmetrical intermediate is excluded, such as oxidation to diacetyl and reduction to acetylmethylcarbinol.

Does this mean then that there are three mechanisms of acetylmethylcarbinol formation, or does it mean that each preparation contains other enzyme systems capable of activating other substances that ultimately are transformed into the intermediates? That is, does the pig heart have enzymes that can form the intermediate directly from the synthetic acetaldehyde, possibly a very strong phosphorylating mechanism, which the yeast and bacterial preparation do not have? The yeast preparation, on the other hand, may be able to combine pyruvic acid and acetaldehyde, as suggested by Dirschel (1930) before decarboxylations, while the bacterial preparation does not have an enzyme that can activate synthetic acetaldehyde, but can form the intermediate from the pyruvate. These are questions that await further investigation.

The problem of the distribution of the heavy carbon in the acetylmethylcarbinol formed by the yeast juice lends itself to some speculation. If there were random selection of the acetaldehyde in the simple condensation of two acetaldehyde molecules to form the carbinol, then the heavy carbons should be equally distributed among the carbons of the acetylmethylcarbinol molecule. Some data obtained indicate this to be the case but other data indicate a greater per cent of heavy carbon in the carbinol half of the molecule. This would give support to the Dirschel (1930) scheme that there was a combination of the synthetic acetaldehyde and pyruvate prior to decarboxylation,

then a reduction or an oxidation to a symmetrical molecule, such as 2,3-butylene glycol or diacetyl, in the process of purification and degradation. This might well account for the apparent discrepancy in the results obtained by the different methods of degradation.

SUMMARY AND CONCLUSIONS

The bacterial enzyme cell-free preparation of Silverman and Werkman (1941) does not utilize synthetic heavy carbon acetaldehyde in the formation of acetylmethylcarbinol in the fermentation of pyruvic acid. Synthetic heavy carbon acetaldehyde, prepared in this laboratory following a modification of the method of Cramer and Kistiakowsky (1941), when added to a fermentation of pyruvic acid was all recovered unchanged. The acetylmethylcarbinol contained only the normal amount of heavy carbon. The observations of Silverman and Werkman (1941) are confirmed.

Yeast juices, prepared from baker's and brewer's dried yeast, utilized the heavy carbon acetaldehyde in the formation of acetylmethylcarbinol.

The acetylmethylcarbinol formed in the fermentation of pyruvic acid contained an increase in heavy carbon. Thus the early observations of Neuberg and later those of Green are confirmed by the use of heavy carbon as a tracer element.

The greater the concentration of the yeast juice, the larger the amount of CO_2 and acetylmethylcarbinol formed. Regardless of the enzyme concentration the CO_2-production stopped after 120 minutes. Freezing the juice decreased only slightly the rate of CO_2-production

but the total amount of CO_2 was considerably decreased. The addition of acetaldehyde increased the initial rate of CO_2 production in the low concentration of pyruvate but the reverse was true in the greater concentration.

The presence of acetaldehyde increased the amount of acetylmethylcarbinol formation by the yeast juice.

A very active acetylmethylcarbinol-forming enzyme preparation was obtained from fresh pig heart. This preparation formed acetylmethylcarbinol from acetaldehyde alone. The carbinol was formed without CO_2 production. These investigations support the observations of Neuberg of the existence of carboligase. The addition of acetaldehyde to a pyruvate fermentation by this preparation increased the production of CO_2 during the first ten minutes of the experiment. This confirms the observations of Green et al. (1942).

The enriched heavy carbon acetylmethylcarbinol formed by the yeast juice was degraded and heavy carbon was found in all four carbons. The explanation of the uneven distribution of the heavy carbon in the acetylmethylcarbinol molecule in some experiments requires further study.

Many yeast preparations, fresh and dried, were investigated to determine their ability to form acetylmethylcarbinol. Only one dried preparation, incubated some time at 37° C., showed any promise of yielding an enzyme preparation that would produce a good yield of the carbinol.

An apparatus is described for collecting CO_2 in CO_2-free NaOH and weighing it. This CO_2 is liberated directly into an extraction bulb for analysis on the massspectrometer.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. C.H. Werkman for the helpful suggestions and assistance given during the course of this work and to the other members of the department whose discussion and cooperation have been invaluable.

LITERATURE CITED

Barritt, Maxwell M.
1936. The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. J. Path. Bact. 42: 441-454.

Barritt, Maxwell M.
1937. The origin of acetylmethylcarbinol in bacterial fermentation. J. Path. Bact. 44: 679-690.

Beams, J.W.
1930. An apparatus for obtaining high speeds of rotation. Rev. Sci. Instruments 1: 667-671.

Beynum, J. van and Pette, J.W.
1939. The decomposition of citric acid by Betacoccus cremonis. J. Dairy Research 10: 250-266.

Booth, V.H. and Green, D.E.
1938. A wet crushing mill for microorganisms. Biochem. J. 32: 855-861.

Brockmann, M.C. and Werkman, C.H.
1933. Determination of 2,3-butylene glycol in fermentations. Ind. Eng. Chem. Anal. Ed. 5: 206-207.

Cramer, R.D. and Kistiakowsky, G.B.
1941. The synthesis of radioactive lactic acid. J. Biol. Chem. 137: 549-555.

Desmots, H.
1904. Production de l'acetylmethylcarbinol par les bacteries du groupe du Bacillus mesentericus. Compt. Rend. Acad. Sci. 138: 581-583.

Dirscherl, Wilhelm and Braun, Emil.
1930. Zur Kenntnis des Acetoins und seiner Dimere. (1 Mitteil. über Acyloine). Ber. Deutsch. Chem. Ges. 63B: 416-422.

Dirscherl, Wilhelm.
1930. Die Bildung von Acetoin aus Acetaldehyd und aus Brenztraubensäure durch Bestrahlung mit ultraviolettem Licht. II. Mitteilung über Acyloine. Z. physiol. Chem. 188: 225-246.

Dirscherl, Wilhelm.
1931. Mechanismus und Kinetik der Acyloinbildung bei der Gärung. III. Mitteilung über Acyloine. Z. physiol. Chem. 201: 47-77.

Dirscherl, Wilhelm, and Schöllig A.
1938. Isolierung und optische Aktivität des bei der Vergärung von Brenztraubensaure mit Frischhefe, Trockenhefe und Macerationssaft gebildeten Acetoins. 6. Mitteilung über Acyloine. Z. physiol. Chem. 252: 53-69.

Dirscherl, Wilhelm and Schöllig, Alfred.
1938. Die Nichtexistenz der Carboligase. 7. Mitteilung über Acyloine. Z. physik. Chem. 252: 70-75.

Dirscherl, Wilhelm, and Nahm, H.
1940. Nichtfermentative Decarboxylierung der Brenztraubensäure und Acetoinbildung. 9. Mitteilung über Acyloine. Z. physiol. Chem. 264: 41-56.

Dixon, M.
1934. Manometric methods. Cambridge Univ. Press.

Donker, H.J.L.
1926. Bijdrage tot de kennis der boterzuurbutylalcoholen acetongistingen. W.D. Meinema, Delft, Holland.

Donnally, L.H.
1933. Quantitative determination of formaldehyde and benzaldehyde and their bisulfite addition products. Ind. Eng. Chem. Anal. Ed. 5: 91-92.

Elion, L.
1926. Zur Kenntnis der Acetoinbildung bei der Vergärung von Zucker durch Hefe. Biochem. Z. 169: 471-477.

Elion, L.
1926. Über die Bildung von Acetaldehyd und Acetymethylcarbinol bie der Gärung und der Atmung der Hefe. Biochem. Z. 171: 40-44.

Friedemann, T.E. and Kendall, A.I.
1929. The determination of lactic acid. J. Biol. Chem. 82: 23-55.

Friedemann, T.E. and Graeser, J.B.
1933. The determination of lactic acid. J. Biol. Chem. 100: 291-308.

Gorr, Gunther und Wagner, Joachim.
1932. Ist die carboxylatische Spaltung der Brenztraubensäure in Acetaldehyd und Kohlensäure durch Leberbrei eindeutig erwiesen? Biochem. Z. 254: 5-7.

Gorr, Gunther.
1932. Über tierische Carboligase. Biochem. Zeit. 254: 12-14.

Green, D.E., Westerfeld, W.W., Vennesland, Birgit and Knox, W.E.
1942. Carboxylases of animal tissues. Jour. Biol. Chem. 145: 69-84.

Gross, N.H., Wood, H.G. and Werkman, C.H.
1942. Fixation of heavy carbon acetaldehyde by active juices. J. Bact. 44: 257.

Hammer, B.W.
1931. Importance of acetylmethylcarbinol and biacetyl in butter culture. Iowa Agricultural Experiment Station, Report Agr. Research 1930/31: 57.

Hammer, B.W.
1934. Products formed by Streptococcus citrovorus and Streptococcus paracitrovorus from citric acid and from lactic acid. Iowa Agricultural Experiment Station, Report Agr. Research 1933/34: 86-87.

Hammer, B.W., Stahly, G.L., Werkman, C.H. and Michaelian, M.B.
1935. Reduction of acetylmethylcarbinol and biacetyl to 2,3-butyleneglycol by the citric acid-fermenting streptococci of butter cultures. Ia. Agr. Exp. Sta. Res. Bull. 191: 381-407.

Hammer, B.W.
1936. The action of aldehydes on certain cultures of Streptococcus liquefaciens. J. Bact. 31: 479-488.

Harden, A. and Walpole, G.S.
1905. Chemical action of Bacillus lactis aerogenes (Escherich) on glucose and mannitol: Production of 2,3-butylene glycol and acetylmethylcarbinol. Proc. Roy. Soc. (London) B. 77: 399-405.

Harden, A. and Norris, Dorothy.
1912. The bacterial production of acetylmethylcarbinol and 2,3-butylene glycol from various substances. Proc. Roy. Soc. (London) B. 84: 492-499.

Harden, A. and Norris, D.
1912. II. The bacterial production of 2,3-butylene glycol and acetylmethylcarbinol from various substances. Proc. Roy. Soc. (London) B. 85: 73-78.

Hermann, S. and Neuschul, P.
1932. Milchsäure und Brenztraubensäure Abbau durch Essigbakterien. Biochem. Z. 246: 446-459.

Hirsch, Julius.
1922. Uber eine biosynthetische Kohlenstoffkettenverknupfung in der aliphatischen Reihe. Zur Kenntnis der Carboligase. Biochem. Z. 131: 178-187.

Horowitz, Wlassowa, L.M. and Rodionowa, E.A.
1933. Über die Azetoingarung. Zentr. Bakt. Parasitenk, II. 87, 333-339.

Johnson, M.J., Peterson, W.H. and Fred, E.B.
1933. Intermediary compounds in the acetone-butyl alcohol fermentation. J. Biol. Chem. 101: 145-157.

Kluyver, A.J. and Donker, H.J.L.
1924. The formation of acetylmethylcarbinol and 2,3-butylene glycol in the fermentation of sugars by alcohol yeasts and true lactic acid bacteria. Proc. Neder. Akad. Wetensch. 28: 314-317. Original not seen. Abstract in Chem. Abst. 19: 35. 1925.

Kluyver, A.J., Donker, H.J.L. and Vissert Hooft F.
1925. Über die Bildung von Acetylmethylcarbinol und 2,3-Butylene Glycol in Stoffwechsel der Hefe. Biochem. Z. 161: 361-378.

Lafon, M.
1932. Sur l'origine possible de l'acetylmethylcarbinol dans la degradation du saccharose par le Baccilus subtilis. Bull. Soc. Chim. Biologique 14: 263-271.

Langlykke, A.F. and Peterson, W.H.
1937. Determination of acetylmethylcarbinol. Ind. Eng. Chem. Anal. Ed. 9: 163-166.

Lemoigne, M.
1919. Fermentation butylene-glycolique des sucres par la bacteridie charbonneuse. Compt. Rend. Soc. Biol. 82: 984-986.

Lemoigne, M.
1923. Fermentation butylene-glycolique du glucose par certaines bacteries du group du Bacillus proteus. Compt. Rend. Soc. Biol. 88: 467-468.

Lemoigne, M. and Monguillon, P.
1930. Presence de l'acetylmethylcarbinol et du 2,3-butylene glycol chez les plantes superieures. Formation au cours de la germination. Compt. Rend. Acad. Sci. 190: 1457-1459.

Lemoigne, M. and Monguillon, P.
1930. Presence de l'acetylmethylcarbinol et du, 2,3-butylene-glycol dans le sang des animaux superieurs. Compt. Rend. Acad. Sci. 191: 80-82.

Levine, M.
1921. Bacteria fermenting lactose and their significance in water analysis. Iowa Eng. Exp. Sta. Bull. 62.

Lipmann, F.
1937. Die Dehydrierung der Brenztraubensäure. Enzymologia 4: 65-72.

Mashing, T.
1936. Chemical determination of bread aroma. Mühlenlab. 6: 90-94. Original not seen. Abstract in Chem. Abst. 31, 7542.

Michaelian, M.B., Farmer, R.S. and Hammer, B.W.
1933. Relationship of acetylmethylcarbinol and biacetyl to butter cultures. Iowa Agr. Exp. Sta. Res. Bull. 155: 325-360.

Mickelson, M.N. and Werkman, C.H.
1938. Influence of pH on the dissimilation of glucose by Aerobacter indologenes. J. Bact. 36: 67-76.

Mickelson, M.N.
1939. Intermediary metabolism of coli-aerogenes bacteria. Ph.D. thesis. Iowa State College.

Mickelson, M.N. and Werkman, C.H.
1939. Effect of aldehydes and fatty acids as added hydrogen acceptors on the fermentation of glucose by Aerobacter indologenes. J. Bact. 37: 619-628.

Mickelson, M.N. and Werkman, C.H.
1939. Pressure-aeration effects on the dissimilation of glucose by Aerobacter indologenes. Iowa State College J. Sci. 13: 157-160.

Nagelschmidt, G.
1927. Gestufte phytochemische Reduktion. Biochem. Z. 186: 317-321.

Neuberg, C. and Reinfurth, Elsa.
1918. Die Festlegung der Aldehydestufe bei der alkoholischen Gärung. Ein experimenteller Beweis der Acetaldehyde - Brenztraubensäure-theorie. Biochem. Z. 89: 365-414.

Neuberg, C., Nord, F.F. and Wolff, E.
1920. Acetaldehyde als Zwischenstufe bei der Vergärung von Zucker durch B. lactic aerogenes. Biochem. Z. 112: 144-150.

Neuberg, C. and Hirsch, J.
1921. Über ein Kohlenstoffketten knüpfendes Ferment (Carboligase) Biochem. Z. 115: 282-310.

Neuberg, C. and Ohle, H.
1922. Zur Kenntnis der Carboligase IV. Mitteilung. Weiter Feststellungen über die biosynthetische Kohlenstuffkettenverknüpfung beim Gärungsvorgange. Biochem. Z. 128: 610-618.

Neuberg, C. and May, A.V.
1923. Die Bilanz der Brenztraubensäuregärung. Biochem. Z. 140: 299-314.

Neuberg, C. and Reinfurth, E.
1923. Eine neue Form der Umwandlung des Acetaldehyd durch gärende Hefe. VI. Mitteilung Über Carboligase. Biochem. Z. 143: 553-565.

Neuberg, C. and Rosenthal, O.
1924. Zusammenhang von carboligatischer Synthese mit carboxylatischem Abbau. (Verwendung von Acetaldehyd als Abfangmittel) Ber. Deutsch Chem. Ges. 57B, 1436-41.

Neuberg, C. and Simon, E.
1925. Zur Kenntnis der biochemischen Acyloinsynthesis. VIII. Mitteilung über Carboligase. Biochem. Z. 156: 374-378.

Neuberg, C. and Kobel, M.
1925. I. Über das physiologische Verhalten des Acetoin. Biochem. Z. 160: 250-255.

Neuberg, C. und Gottschalk, A.
1925. Über den Nachweis von Acetaldehyd als Zwischenstufe bei der anaeroben Atmung höherer Pflanzen. Biochem. Z. 160: 256-260.

Neuberg, C. and Kobel, M.
1929. Carboligase. In Oppenheimer, C. Die Fermente und ihre Wirkungen. G. Thieme, Leipzig, 5th Edition, 3: 1329-37.

Nier, A.O. and Gulbransen, E.A.
1939. Variations in the relative abundance of the carbon isotopes. J. Am. Chem. Soc. 61: 697-698.

Nier, A.O.
1940. A mass spectrometer for routine isotope abundance measurements. Rev. Sci. Inst. 11: 212-216.

Nier, A.O. and Bardeen, J.
1941. The production of concentrated carbon (13) by thermal diffusion. J. Chem. Phys. 9: 690-692.

O'Meara, R.A.Q.
1931. A simple, delicate and rapid method of detecting the formation of acetylmethylcarbinol by bacteria fermenting carbohydrates. J. Path. Bact. 34: 401-406.

Osburn, O.L. and Werkman, C.H.
1932. Determination of carbon in fermented liquors. Ind. Eng. Chem. Anal. Ed. 4: 421-423.

Peters, R.A.
1938. The catalorulin test for vitamin B_1. Biochem. J. 32: 2031-2036.

Pritzker, J., and Jungkunz, Rob.
1930. Extrakt- und Zuckerbestimmung in Wein - und Obstweinessig unter Berücksichtigung des Acetylmethylcarbinols. Mitt. Lebensm. Hyg. 21: 354-362.

Reynolds, Howard.
1936. The dissimilation of carbohydrates by the colon-aerogenes bacteria. Iowa State Coll. Jr. Sci. 11: 97-99.

Reynolds, H. and Werkman, C.H.
1937. The intermediate dissimilation of glucose by Aerobacter indologenes. J. Bact. 33: 603-614.

Reynolds, H., Jacobsson, B.J. and Werkman, C.H.
1937. The dissimilation of organic acids by Aerobacter indologenes. J. Bact. 34: 15-20.

Reynolds, H. and Werkman, C.H.
1937. Carboligatic activity of Escherichia coli. Archiv fur Mikrobiologie 8: 149.

Schmalfuss, Hans, von Hiusch, W., und Schmalfuss, H.
1930. Diacetyl und Methyl-acetyl-carbinol in menschlichem Harn? Z. Physiol. Chem. 188: 279-280.

Schmalfuss, Hans.
1934. Diacetyl und Methyl-acetyl-carbinol (oder ihre kochunbestandigen Vorstufen) im Blut. Z. Physiol. Chem. 227: 247-250.

Silverman, M. and Werkman, C.H.
1940. Acetylmethylcarbinol enzyme system of Aerobacter aerogenes. Proc. Soc. Exptl. Biol. Med. 43: 777-778.

Silverman, M. and Werkman, C.H.
1941. The formation of acetylmethylcarbinol from pyruvic acid by a bacterial enzyme preparation. J. Biol. Chem. 138: 35-48.

Silverman, M.
1941. Microbial formation of acetylmethylcarbinol. Ph.D. thesis, Iowa State College.

Slade, H.D. and Werkman, C.H.
1941. The anaerobic dissimilation of citric acid by cell suspensions of Streptococcus paracitrovorus. J. Bact. 41: 675-684.

Slade, H.D. and Werkman, C.H.
1942. Bacterial assimilation of acetic and succinic acids containing heavy carbon by Aerobacter indologenes. Proc. Soc. Exptl. Biol. Med. 51: 65-66.

Stahly, G.L., Hammer, C.W., Michaelian, M.B. and Werkman, C.H.
1935. The fate of acetylmethylcarbinol and biacetyl in dairy products. Proc. Iowa Acad. Sci. 42: 73-76.

Stahly, G.L. and Werkman, C.H.
1936. Determination of acetylmethylcarbinol in fermentation liquors. Iowa State College J. Sci. 10: 205-211.

Stahly, Grant L.
1936. Dissimilation of carbohydrates by bacteria of the genus Aerobacillus. Iowa State Coll. Jr. Sci. 11: 110-112.

Stahly, G.L. and Werkman, C.H.
1942. Origin and relationship of acetylmethylcarbinol to 2,3-butylene glycol in bacterial fermentations. Biochem. J. 36: 575-581.

Stepanov, A. and Kuzin, A.
Über die Synthese einer Kohlen-stoffkette mit Hilfe von Enzymen. V. Mitteil: Vorkommen der Carboligase im Tier-organismus. Ber. Deutsch. Chem. Ges. 67B: 721-722.

Tanko, B. and Munk, L.
1939. Beiträge zum Carboligaseproblem. Z. Physiol. Chem. 262: 144-157.

Tanko, B., Munk, L., and Abonyi, I.
1940. Über die optische Aktivität der durch biochemische Synthese entstandenen Acetoine. Z. Physiol. Chem. 264: 91-107.

Tomiyasu, Yuko.
1936. Existence of carboligase. Biochem. Z. 289: 97-103 J. Agr. Chem. Soc. Japan 12: 1132-40. Chem. Abst. 31: 2240. (1937).

van Niel, C.B., Kluyver, A.J. and Derx, H.B.
1929. Über das Butteraroma. Biochem. Z. 210: 234-251.

Verhave, T.H.
1928. 2,3-Butylene glycol. Brit. Patent 315, 263 and Brit. Patent 315-264. C.A. 24: 1652.

Virtanen, Artturi, I. Kontio, Pekka and Storgards, Torsten.
1940. Aroma formation in butter. Suomen Kemistilehti 13B: 23 (In German) Original not seen. Abstract in Chem. Abst. 35: 2622 (1941).

Visser't Hooft, F. and de Leeuw, F.J.G.
1935. The occurrence of acetylmethylcarbinol in bread and its relation to bread flavor. Cereal chem. 12:213-229.

Walpole, G.S.
1910. The action of B. lactis aerogenes on glucose and mannitol. II. The investigation of the 2,3-butandiol and the acetylmethylcarbinol formed: the effect of free oxygen on their production: the action of the B. lactis aerogenes on glucose. Proc. Roy. Soc. (B) London, 83: 272-286.

Werkman, C.H.
1930. An improved technic for the Voges-Proskauer test. J. Bact. 20: 121-125.

Werkman, C.H.
1939. Bacterial dissimilation of carbohydrates. Bact. Rev. 3: 187-227.

Wiggert, W.P., Silverman, M., Utter, M.F. and Werkman, C.H.
1940. Preparation of an active juice from bacteria. Iowa State Coll. J. Sci. 14: 179-186.

Wilson, P.W., Peterson, W.H. and Fred, E.B.
1927. The production of acetylmethylcarbinol by Clostridium acetobutylicum. J. Biol. Chem. 74: 495-507.

Yamada, Masakazu and Kurono, Kanyoku.
1927. Production of acetoin and 2,3-butylene glycol by microbes and their distribution in fermentation products. Bull. Agr. Chem. Soc. Japan 3: 83-86. Original not seen. Abstract in Chem. Abst. 22: 841. (1928).